Aristotle University of Thessaloniki
Faculty of Sciences
School of Biology
Department of Ecology

PhD Dissertation

**Airborne pollen patterns
along an altitudinal gradient of the Italian Alps**

*combination of classical pollen identification methods and
next generation sequencing on environmental DNA*

Kleopatra Leontidou

Thessaloniki 2018

Aristotle University of Thessaloniki
Faculty of Sciences
School of Biology
Department of Ecology

PhD Dissertation

Airborne pollen patterns
along an altitudinal gradient of the Italian Alps

combination of classical pollen identification methods
and next generation sequencing on environmental DNA

Kleopatra Leontidou

Thessaloniki 2018

Αριστοτέλειο Πανεπιστήμιο Θεσσαλονίκης
Σχολή Θετικών Επιστημών
Τμήμα Βιολογίας
Τομέας Οικολογίας

Διδακτορική Διατριβή

Πρότυπα αερομεταφερόμενης γύρης κατά μήκος μιας υψομετρικής διαβάθμισης στις Ιταλικές Άλπεις

εφαρμογή αλληλούχισης νέας γενιάς σε περιβαλλοντικό DNA και κλασικών μεθόδων ταυτοποίησης γύρης

Κλεοπάτρα Λεοντίδου

Θεσσαλονίκη 2018

Examination committee

Despoina Vokou[1,2], Professor

School of Biology, Aristotle University of Thessaloniki

Maurizio Casiraghi[2], Associate professor

School of Biotechnology and Biosciences, University of Milano-Bicocca (Italy)

Cristiano Vernesi[2], Researcher

 Fondazione Edmund Mach (Italy)

Athanasios Damialis[3], Researcher

UNIKA-T (Germany)

Andreas Drouzas[3], Assistant professor

School of Biology, Aristotle University of Thessaloniki

Athanasios Kallimanis[3], Associate professor

School of Biology, Aristotle University of Thessaloniki

Ioannis Tsiripidis[3], Assistant professor

School of Biology, Aristotle University of Thessaloniki

----

[1] Supervisor

[2] Members of the advisory committee

[3] Members of the examination committee

Εξεταστική Επιτροπή

Δέσποινα Βώκου[1,2], Καθηγήτρια
Τμήμα Βιολογίας, Αριστοτέλειο Πανεπιστήμιο Θεσσαλονίκης

Maurizio Casiraghi[2], Αναπληρωτής καθηγητής
Τμήμα Βιοτεχνολογίας και Βιοεπιστημών, University of Milano-Bicocca (Ιταλία)

Cristiano Vernesi[2], Ερευνητής
Fondazione Edmund Mach (Ιταλία)

Αθανάσιος Δάμιαλης[3], Ερευνητής
UNIKA-T (Γερμανία)

Αντρέας Δρούζας[3], Επίκουρος καθηγητής
Τμήμα Βιολογίας, Αριστοτέλειο Πανεπιστήμιο Θεσσαλονίκης

Αθανάσιος Καλλιμάνης[3], Αναπληρωτής καθηγητής
Τμήμα Βιολογίας, Αριστοτέλειο Πανεπιστήμιο Θεσσαλονίκης

Ιωάννης Τσιριπίδης[3], Επίκουρος καθηγητής
Τμήμα Βιολογίας, Αριστοτέλειο Πανεπιστήμιο Θεσσαλονίκης

----

[1] Επιβλέπουσα
[2] Μέλη της συμβουλευτικής επιτροπής
[3] Μέλη της εξεταστικής επιτροπής

Η έγκρισις της παρούσης διπλωματικής εργασίας υπό του Τμήματος Βιολογίας της Σχολής
Θετικών Επιστημών του Αριστοτελείου Πανεπιστημίου Θεσσαλονίκης δεν υποδηλοί αποδοχήν των γνωμών του συγγραφέως (Ν.5343/1932, άρθρ. 202, παρ. 2).

'Always keep Ithaca in mind'

To everybody in my 'journey'

Σα βγεις στον πηγαιμό για την Ιθάκη,
να εύχεσαι να 'ναι μακρύς ο δρόμος,
γεμάτος περιπέτειες, γεμάτος γνώσεις.
...
Πάντα στο νου σου να 'χεις την Ιθάκη.
Το φθάσιμον εκεί είν' ο προορισμός σου.
Αλλά μη βιάζεις το ταξίδι διόλου.
Καλλίτερα χρόνια πολλά να διαρκέσει.
...
Κι αν πτωχική την βρεις, η Ιθάκη δεν σε γέλασε.
Έτσι σοφός που έγινες, με τόση πείρα,
ήδη θα το κατάλαβες οι Ιθάκες τι σημαίνουν.

(Ιθάκη, Κ. Π. Καβάφης)

As you set out bound for Ithaka
hope that the journey is a long one,
full of adventure, full of learning.
...
Always keep Ithaca in mind.
To arrive there is your destination.
But in no way rush the voyage.
...
And though you may find her wanting, Ithaka has not deceived you.
Wise as you 've become, with so much experience,
already you will have understood what these Ithakas mean.

(Ithaca, C. P. Cavafy)

Acknowledgements

Thanks to many people, this doctoral thesis reached its final destination, after a long journey. In practice, I wish that this is an academic work that will contribute something, even small, to the existing knowledge. Apart from the professional completion of this work, I feel I achieved a personal goal as well: to learn, improve and evolve.

I wish to thank my supervisor Prof. Despoina Vokou for her constructive guidance and trust all these years. Our discussions always had something to give me as a student. With her substantial advices and all the knowledge she managed to transmit, she gave me important motivation to keep on improving on scientific and personal level.

For supporting this collaborative work, I wish to thank the other two members of the advisory committee, Prof. Maurizio Casiraghi from the University of Milano-Bicocca and Dr. Cristiano Vernesi from Fondazione Edmund Mach. For her collaboration, I thank also Antonella Cristofori from Fondazione Edmund Mach. I thank all of them for the opportunities they provided within BioAir project, from the working environment in Italy to the implementation of the project in the Italian Alps.

I would also like to thank all the members of the examination committee, Dr. Athanasios Damialis, Prof. Andreas Drouzas, Prof. Athanasios Kallimanis and Prof. Ioannis Tsiripidis for all their useful suggestions that improved the text of the thesis.

For their collaboration, I thank all the members of Conservation Genetics group and Ecogenomics group from Fondazione Edmund Mach, the members of ZooPlant lab group of Milano-Bicocca University for hosting me in their lab and all colleagues in Italy and Greece for any contribution, useful discussions and kind help in the work.

Special thanks to Maria Cristina Viola from Fondazione Edmund Mach for her direct response to all the needs of this work and her kind support in the lab and field work all these years and Matteo Girardi from Fondazione Edmund Mach, who provided technical support and important help in the lab. Finally, I thank the National Park of Paneveggio - Pale di San Martino for hosting us there and in particular Maurizio Salvadori who helped in the field work and guided us in the beautiful mountains of Dolomites and the Aerobiological center of San Michele all'Adige, in particular Elena Gottardini, for providing aerobiological monitoring data.

Thank you all!

The photo of the book cover (front and back) is taken in Paneveggio National Park, kindly provided from Carlo A. Turra.

Table of Contents

Table of Contents (continued)

Brief Abstract

The aim of the study was (i) the development of an efficient method for pollen taxonomic identification from complex environmental samples by use of molecular approaches, and (ii) the application of this method for the detection of spatio-temporal patterns of airborne pollen in a mountainous area and the evaluation of the potential of metabarcoding to be used for monitoring vegetation changes. We developed an optimal protocol for processing samples of different type and complexity for DNA extraction, further applied Sanger and next generation sequencing (NGS), and compared results to those taken by classical microscopy. We applied this protocol in combination with NGS to environmental samples from six habitats along the altitudinal gradient of the Italian Alps (National Park of Paneveggio–Pale di San Martino). Providing high resolution taxonomic results, metabarcoding can be used for biodiversity assessments and floral surveys and for monitoring vegetation changes, particularly those expressed in species composition rather than in species abundance.

Σύντομη Περίληψη

Σκοπός της διατριβής ήταν (α) η ανάπτυξη κατάλληλης μεθοδολογίας ανίχνευσης και ταυτοποίησης γύρης σε περιβαλλοντικά δείγματα με εφαρμογή μοριακών προσεγγίσεων και (β) η εφαρμογή αυτής της μεθοδολογίας για την ανίχνευση χωρικών και χρονικών προτύπων της αερομεταφερόμενης γύρης σε μια υψομετρική διαβάθμιση και τη διερεύνηση της δυνατότητας να χρησιμοποιηθεί η προσέγγιση metabarcoding για την βιοπαρακολούθηση των αλλαγών της βλάστησης. Αναπτύχθηκε κατάλληλο πρωτόκολλο επεξεργασίας δειγμάτων διαφορετικού τύπου και πολυπλοκότητας προς απομόνωση του DNA γύρης, χρησιμοποιήθηκαν οι μέθοδοι αλληλούχισης Sanger και νέας γενιάς (NGS) και συγκρίθηκαν τα αποτελέσματα που προέκυψαν με τα προερχόμενα από την κλασική μέθοδο ταυτοποίησης γύρης με μικροσκόπιο. Το πρωτόκολλο αυτό σε συνδυασμό με τη μέθοδο NGS εφαρμόστηκε σε περιβαλλοντικά δείγματα από έξι οικοτόπους των Ιταλικών Άλπεων (εθνικό πάρκο Paneveggio–Pale di San Martino) ενώ κατασκευάστηκε μια τοπική βάση δεδομένων με αλληλουχίες DNA για τα taxa γύρης της περιοχής. Παρέχοντας υψηλής ανάλυσης αποτελέσματα, η προσέγγιση metabarcoding μπορεί να εφαρμοστεί για εκτίμηση της φυτικής βιοποικιλότητας και για την παρακολούθηση αλλαγών στη βλάστησης, κυρίως αυτών που αφορούν στη σύνθεση παρά στην αφθονία των ειδών.

Abstract

With the combination of classical pollen identification methods and next generation sequencing on environmental DNA, airborne pollen patterns were investigated in six habitats along an altitudinal gradient of Pale di San Martino-Paneveggio National Park (Italian Alps). Pollen was identified from environmental samples that were collected with gravimetric Tauber traps. Results of both methods were evaluated to investigate how similarly they can capture pollen spatio-temporal patterns and how they can be used for different applications, such as for diversity assessment and monitoring studies.

For the taxonomic identification of pollen DNA, a reference database of DNA sequences was first constructed. TrnL sequences were downloaded from Genbank for most of the anemophilous taxa present in the study area (Trentino, northern Italy) and they were processed and stored in a local database. For plant species without available sequences, plant material was collected, the trnL gene was targeted and sequenced and the new sequences were integrated in the final database, in total 1188 sequences corresponding to 403 species of 198 genera and 46 families; from these 44 were new sequences, corresponding to 26 species.

Preliminary experiments for metabarcoding analysis of airborne pollen were designed in order to develop appropriate lab procedures. The experimental design included sample collection from volumetric and gravimetric traps at the Aerobiological Monitoring Centre of Fondazione Edmund Mach, which were utilized both for microscope and molecular analysis. Experiments were performed for the steps of sample processing to retrieve pollen pellets and of DNA extraction of optimal yield. The protocols were applied across a complexity of samples from single-species pollen to environmental multiple-species samples. It was found that a number of factors like the mechanic disruption of the pollen cell walls or the extraction kit used influence significantly the DNA yield. On the basis of the results taken, the optimal combination was selected (Nucleomag kit and disruption with steel beads). Also, it was found that the DNA yield decreases after chemical treatment of the sample, which suggests that better washing of the pollen pellet

should be applied instead.

A small fragment (about 150 base pairs) of the chloroplast trnL gene was amplified and sequenced (with cloning and classic Sanger sequencing) using universal primers for plants. For the taxonomic identification we used a custom trnL database as reference. The results showed that 75% and 37% of the taxa identified by microscope were revealed by metabarcoding for the volumetric and the gravimetric samplings, respectively. The whole procedure allowed the identification of pollen from environmental samples. The taxonomic information showed higher resolution via metabarcoding, while the amount and quality of information could increase with the application of next generation sequencing.

Having defined the optimal methodology, we could proceed to the analysis of the samples from the park (54 in total). Sequencing was performed this time with the Illumina next generation sequencing platform. The sequencing data resulted in a total of 11,137,178 sequences, clustered in 140 Operational Taxonomic Units which were assigned taxonomically to 32 families, 55 genera (or group of genera). According to the plant growth form, 37 taxa represent woody and 25 herbaceous plants. Thirteen of these species (21%) are not present in the plant checklist of the park; *Cedrus* and *Cupressus sempervirens* pollen, in particular, had a considerable contribution of >1%. There are 13 main pollen taxa contributing at least 0.5% to the total number of the sequence reads. These are *Pinus* (36.8%), *Larix decidua* (14.5%), *Cedrus* (12.4%), *Picea* (11.6%), *Abies* (5.4%), *Corylus/Ostrya/ Carpinus* (5%), *Alnus viridis* (2.9%), *Urtica dioica* (2.8%), *Juniperus communis* (0.7%), *Taxus baccata* (0.6%), *Chenopodium album* (0.6%), *Festuca/Trisetum/Lolium* (0.5%) and *Cupressus sempervirens* (0.5%).

When we used concurrently metabarcoding and classical microscopic analysis of the pollen trapped, almost all non-rare families were commonly identified by the two methods, but the molecular method could discern more genera. Nevertheless, Cyperaceae and Polygonaceae, although with considerable abundance in the microscopic dataset, did not feature in the metabarcoding results. Compared to the total pollen recorded, Poaceae Betulaceae, Corylaceae and Oleaceae were found to contribute less with the

metabarcoding method than with the microscopic one, and Pinaceae more. For the main pollen season, *Pinus* is the most abundantly represented taxon in the pollen spectrum after both methods and similarly at high concentrations in the aerobiological data (Lanzoni sampler). Nevertheless, its contribution to the park's vegetation is lower than that of the dominant *Picea* (85%), the pollen of which consists only 12% of the annual sum.

Regarding the biodiversity assessment, metabarcoding could discern the sampling periods. It detected March-July 2015 as the period with the highest number of taxa (alpha diversity), and revealed significant changes in diversity (beta diversity) among sampling periods. These results matched the features of the pollen season, as defined by aerobiological studies running in parallel. Spatial patterns could not be clearly defined; nevertheless, results of metabarcoding were in accordance to the ones obtained with the microscopic method.

Optimized molecular protocols can increase our potential for time-efficient analysis of pollen datasets. Providing high resolution taxonomic results, the molecular method that we applied can be used for biodiversity assessments and floral surveys or for monitoring vegetation changes, particularly those expressed in species composition rather than in species abundance. On the basis of our results and previous reports, we can argue that the metabarcoding and the microscopic methods have each their weak and strong points and they should be applied in a complementary way, at least until the quantitative and qualitative issues associated with metabarcoding are adequately addressed.

Περίληψη

Με συνδυασμό κλασικών μεθόδων ταυτοποίησης γύρης και αλληλούχισης νέας γενιάς σε περιβαλλοντικό DNA, διερευνήθηκαν τα χωρικά και χρονικά πρότυπα της αερομεταφερόμενης γύρης σε μια υψομετρική διαβάθμιση. Συγκεκριμένα έγιναν δειγματοληψίες σε έξι διαφορετικούς οικοτόπους κατά την υψομετρική διαβάθμιση του πάρκου Pale di San Martino-Paneveggio (Ιταλικές Άλπεις). Η γύρη ταυτοποιήθηκε σε δείγματα που συλλέχθηκαν με παγίδες καθίζησης τύπου Tauber. Εξετάστηκαν τα αποτελέσματα που προέκυψαν με βάση την κλασική μικροσκοπική και με τη μοριακή ανάλυση για να αξιολογηθεί πόσο παρόμοια είναι τα χωρο-χρονικά πρότυπα γύρης που ανιχνεύουν και εάν και πώς μπορούν να χρησιμοποιηθούν για διάφορες εφαρμογές, όπως για εκτιμήσεις βιοποικιλότητας και για μελέτες βιοπαρακολούθησης.

Για την ταξινομική αναγνώριση των φυτών που συνεισφέρουν DNA γύρης στα περιβαλλοντικά δείγματα, αρχικά σχεδιάστηκε μια βάση δεδομένων αναφοράς. TrnL ακολουθίες μεταφορτώθηκαν από την Genbank για τα περισσότερα ανεμόφιλα taxa που υπάρχουν στην περιοχή μελέτης (Τρεντίνο, βόρεια Ιταλία) και ακολούθως επεξεργάστηκαν και αποθηκεύτηκαν σε μια τοπική βάση δεδομένων. Για τα πιο συχνά είδη που καταγράφονταν σε αεροβιολογικά δεδομένα της περιοχής, αλλά για τα οποία δεν υπήρχαν αλληλουχίες, συλλέχθηκε φυτικό υλικό, στοχεύθηκε και αλληλουχήθηκε το κομμάτι trnL και οι νέες αλληλουχίες που προέκυψαν ενσωματώθηκαν στην τελική βάση δεδομένων της μελέτης, συνολικά 1188 ακολουθίες που αντιστοιχούν σε 403 είδη από 198 γένη και 46 οικογένειες· από αυτά 44 ήταν οι νέες ακολουθίες, που αντιστοιχούν σε 26 είδη.

Σχεδιάστηκαν και πραγματοποιήθηκαν πειράματα που αφορούσαν την ανεύρεση των καταλληλότερων συνθηκών για την ανάλυση metabarcoding σε αερομεταφερόμενη γύρη. Το πειραματικό σχέδιο περιλάμβανε συλλογή δειγμάτων από παγίδα καθίζησης και από σταθερή ογκομετρική παγίδα από το αεροβιολογικό κέντρο παρακολούθησης του Fondazione Edmund Mach, που χρησιμοποιήθηκαν τόσο

για μικροσκοπική όσο και μοριακή ανάλυση. Πειράματα έγιναν κατά το στάδιο επεξεργασίας των δειγμάτων για την ανάκτηση ιζήματος γύρης, και κατά το στάδιο απομόνωσης του συνολικού DNA με στόχο την ανεύρεση των συνθηκών βέλτιστης απόδοσης. Τα πρωτόκολλα εφαρμόστηκαν σε μια σειρά δειγμάτων αυξανόμενης πολυπλοκότητας, από γύρη μοναδικού είδους έως περιβαλλοντικά δείγματα πολλαπλών ειδών γύρης. Βρέθηκε ότι μεταβλητές όπως η μηχανική διάρρηξη του τοιχώματος γύρης και η τεχνική απομόνωσης (kit) επηρεάζουν σημαντικά την απόδοση DNA, υποδεικνύοντας την επιλογή του βέλτιστου συνδυασμού (Nucleomag kit και διάρρηξη με χάντρες χάλυβα). Επίσης, βρέθηκε οτι η απόδοση DNA μειώνεται μετά από χημική επεξεργασία του δείγματος, γεγονός που υποδεικνύει καλύτερο πλύσιμο του ιζήματος για αύξηση της απόδοσης.

Ένα μικρό κομμάτι (περίπου 150 ζεύγη βάσεων) του χλωροπλαστιδιακού trnL γονιδίου ενισχύθηκε και αλληλουχήθηκε (με κλωνοποίηση και κλασική αλληλούχιση Sanger) χρησιμοποιώντας γενικούς εκκινητές για φυτά. Για την ταξινομική αναγνώριση χρησιμοποιήσαμε γνωστές trnL αλληλουχίες σε βάση δεδομένων ως αλληλουχίες αναφορές. Τελικά, βρέθηκε οτι 75% και 37% των taxa που αναγνωρίστηκαν μέσω μικροσκοπίου ανιχνεύτηκαν και με metabarcoding για τους δυο τύπους παγίδων αντίστοιχα. Η ταξινομική πληροφορία ήταν πιο λεπτομερής επιτρέποντας αναγνώριση και σε χαμηλότερα ταξινομικά επίπεδα, ενώ το μέγεθος της πληροφορίας αυξήθηκε σε μεγάλο βαθμό με την εφαρμογή αλληλούχισης νέας γενιάς.

Αφού προσδιορίστηκε η βέλτιστη μεθοδολογία, εφαρμόστηκε επακόλουθα στα δείγματα του πάρκου (54 συνολικά), στα οποία έγινε αλληλούχιση νέας γενιάς Illumina Miseq. Οι αλληλουχίες που αποκτήθηκαν ήταν συνολικά 11137178 και ομαδοποιήθηκαν σε 140 OTUs που ταξινομήθηκαν σε 32 οικογένειες και 55 γένη (ή ομάδες γενών). Σύμφωνα με την αυξητική μορφή των φυτών, 37 taxa γύρης προέρχονται από ξυλώδη και 25 από ποώδη φυτά. Δεκατρία από αυτά τα είδη (21%) δεν βρέθηκαν στην λίστα ειδών του πάρκου. Μάλιστα, η γύρη των *Cedrus* και *Cupressus sempervirens* είχε αξιόλογη συμμετοχή, >1%. Υπάρχουν 13 κύρια τάξα γύρης που

συμβάλλουν τουλάχιστον 0.5% στο συνολικό αριθμό αλληλουχιών. Αυτά ήταν τα *Pinus* (36,8%), *Larix decidua* (14,5%), *Cedrus* (12,4%), *Picea* (11,6%), *Abies* (5,4%), *Corylus/ Ostrya/Carpinus* (5%), *Alnus viridis* (2,9%), *Urtica dioica* (2,8%), *Juniperus communis* (0,7%), *Taxus baccata* (0,6%), *Chenopodium album* (0,6%), *Festuca/Trisetum/Lolium* (0,5%) και *Cupressus sempervirens* (0,5%).

Η ταυτόχρονη χρήση metabarcoding και μικροσκοπικής ανάλυσης έδειξε ότι σχεδόν όλες οι μη σπάνιες οικογένειες ταυτοποιήθηκαν και με τις δυο μεθόδους, αλλά η μοριακή μέθοδος μπόρεσε να διακρίνει περισσότερα γένη. Παρ' όλα αυτά, τα Cyperaceae και Polygonaceae, αν και με αξιόλογη αφθονία στα δεδομένα του μικροσκοπίου, δεν εμφανίστηκαν στα αποτελέσματα του metabarconding. Σε σύγκριση με τη συνολική γύρη που καταγράφηκε, τα Poaceae, Betulaceae, Corylaceae και Oleaceae βρέθηκαν να συνεισφέρουν λιγότερο με τη μέθοδο metabarcoding παρά με τη μικροσκοπική και τα Pinaceae περισσότερο. Για την κύρια περίοδο κυκλοφορίας γύρης, το *Pinus* είναι το πιο άφθονο τάξον στο φάσμα γύρης και με τις δύο μεθόδους, όπως βρέθηκε και σε αεροβιολογικά δεδομένα (με δειγματολήπτη Lanzoni). Ωστόσο, η συμμετοχή του στη βλάστηση του πάρκου είναι χαμηλότερη από αυτή του κυρίαρχου *Picea* (85%), που συμμετέχει ως γύρη μόνο με 12%.

Ως προς τις εκτιμήσεις της βιοποικιλότητας, η μέθοδος metabarcoding ανέδειξε την περίοδο Μαρτίου-Ιουλίου 2015 ως την πλουσιότερη περίοδο σε αριθμό taxa (άλφα ποικιλότητα), ενώ αλλαγές στην ποικιλότητα (βήτα ποικιλότητα) εντοπίστηκαν σε χρονική παρά σε χωρική κλίμακα. Η ανάλυση metabarcoding μπόρεσε να αποκαλύψει τις χρονικές μεταβολές, οι οποίες συμφωνούν με τα χαρακτηριστικά της εποχής κυκλοφορίας γύρης, όπως προσδιορίστηκαν από τις αεροβιολογικές μελέτες που διεξάγονταν παράλληλα και μπόρεσε να διακρίνει τις περιόδους δειγματοληψίας. Στην κλίμακα του χώρου, δεν μπόρεσε να διακρίνει οικοτόπους και υψόμετρα αλλά αντίστοιχα ήταν και τα αποτελέσματα της μικροσκοπικής μεθόδου.

Παρέχοντας υψηλής ανάλυσης αποτελέσματα, η μοριακή μέθοδος που χρησιμοποιήσαμε μπορεί να εφαρμοστεί για εκτίμηση της βιοποικιλότητας και για βοτανικές έρευνες ή για την παρακολούθηση αλλαγών στη βλάστηση και κυρίως για

αυτές που εκφράζονται σε σύνθεση ειδών, παρά σε αφθονία ειδών. Με βάση τα αποτελέσματα της έρευνάς μας, μπορούμε να υποστηρίξουμε οτι η μέθοδος metabarcoding όπως και η μικροσκοπική μέθοδος έχουν η καθεμιά τα αδύναμα και τα ισχυρά σημεία τους και θα πρέπει να εφαρμόζονται συμπληρωματικά, τουλάχιστον μέχρι να αντιμετωπιστούν επαρκώς τα ζητήματα που σχετίζονται με τη διακριτική ικανότητα του metabarcoding και την εκτίμηση της συμμετοχής του κάθε taxon στα δείγματα.

Chapter A. Research background & Aim

Pollen patterns and their spatio-temporal changes were investigated in six habitat types along an elevation gradient of the eastern Italian Alps. To detect pollen patterns, we used next generation sequencing on pollen DNA from environmental samples, because of its potential to provide more detailed and accurate taxonomic information with improved time-efficiency and also light microscopy, which is the classic pollen identification method.

The aims of this study were: (1) to develop an efficient methodology for pollen taxonomic identification from complex environmental samples, which required the development of a local reference database of DNA sequences for the study area and the development of an optimal protocol for processing aerobiological samples for DNA extraction and metabarcoding analysis and (2) to apply this method in order to detect the spatio-temporal patterns of airborne pollen in a mountainous area and to examine the potential of metabarcoding to be used for monitoring vegetation changes.

The study was conducted in the National Park of Paneveggio – Pale di San Martino. Paneveggio, an EU NATURA 2000 protected area. Because of its status, several conservation and monitoring projects have been implemented there over the last decades, thus providing useful information. Pale di San Martino, which is a big mountainous range of the Park and one of the largest groups of the Dolomites, is also a UNESCO World Heritage site.

Results of this study are expected to contribute to a better understanding of the pollen patterns along an altitudinal gradient, increase our capacity to analyze and interpret pollen datasets given the combination of methods applied to the same samples and also provide a baseline for future monitoring. Apart from their ecological importance, they are also of value for the numerous visitors of the Park who suffer from pollen-induced allergies.

Chapter B. General Introduction

B.1 Pollen and biological characteristics

Function

Spermatophyta, the most recent group in plant evolution, develop flowers during their life cycle. Flowers are plant organs produced for sexual reproduction, as soon as the plant gets vegetatively mature (Bozabalidis 2003). The life cycle of flowering plants is shown in Figure B.1. Pollen grains are biological structures produced from the male reproductive part of the flower and contain the male gamete (Xu et al. 1999, Parducci et al. 2017). So as to achieve the fertilization of the female gamete, the pollen grains need to be transported to the female reproductive organs; this is the procedure that is called pollination.

Pollen is produced in the microsporangium of the anther in angiosperms or of the male cone in gymnosperms and gets transported to the female pistil or female cone respectively (Bozabalidis 2003). The flower has morphological and anatomical differences between angiosperms and gymnosperms and it can appear unique or in groups (inflorescence), depending on the pollination mode. The most common types of pollination include:

- insect-pollination (entomophilous plants)
- wind-pollination (anemophilous plants)
- double strategy (amphiphilous plants)

While the gymnosperms are pollinated exclusively by the wind, most of the angiosperms base their pollination on insects and other animals (Vokou 2009). In particular, it has been reported that 78% of the flowering plants in the temperate and 94% in the tropical zone are insect-pollinated (Ollerton et al. 2011). The differences in their flowers include: shrinking of the flowering parts, elongated filaments of the stamen, flowering before leafing and big production of pollen (small, dry and smooth) in gymnosperms, while most angiosperms have more clear perianth of the flower, which

produces aroma and colour to attract the pollinators (Lewis 1983). There are also species that use both wind and insects for pollination. For example, *Castanea sativa*, due to its flower characteristics and the big production of pollen is mainly characterized as anemophilous, but in many cases it seems that the insects' role is important (Giovanetti & Aronne 2011). Finally, there are plants that do not need the help of any biotic or abiotic factors (Figure B.2). The existing types of pollination according to the source of pollen (self or cross-pollination) and agents that can be possibly involved in the procedure are summarized in Figure B.2.

From the pollen produced and released in the air for pollination, part will be successfully used for fertilization, that will give the seed of the future plant (Bozabalidis 2003) (Figure B.1), while another part will be dispersed in the air and circulate in the atmosphere (Lacey & West 2006) (Figure B.3).

Developmental biology

During the anther development and the developmental stages of pollen, two types of cells are differentiated within the anther: reproductive cells which give rise to pollen grains with meiotic divisions and non-reproductive cells, which form the tissue layers such as epidermis, cortical and tapetal cell layers (Muller & Rieu 2016). The pollen grain consists of a large vegetative cell enclosing a small generative cell, which will give the male gametes (two sperm cells) (Xu et al. 1999). The cytoplasm in pollen grains includes mitochondria and chloroplasts (Sangwan 1987). Therefore, pollen's DNA can be nuclear, mitochondrial (mtDNA) and chloroplast DNA (cpDNA) (Parducci et al. 2017). However, degradation of the plastids in pollen has been reported in cases of plastids' maternal inheritance (Primavesi et al. 2015).

Morphology

Pollen grains can vary greatly in morphology, and this is how, by observing them in the optical microscope, one can distinguish the plant producing them in taxonomic studies

(Moore et al. 1991). Below are summarized the main morphological characters.

Dispersal unit: Pollen grains may be single (monads) or in groups (dyad, tetrad or polyad).

Aperture: It refers to the openings of the exine. There are pollen grains without apertures (inaperturate), like the conifers or others that have apertures (aperturate). This feature is very informative and easy to observe in the microscope. Based on the apertures' characteristics, one can observe: (i) the type of aperture (pores, which are circular openings, or furrows, which are elongated openings, or a combination of these), (ii) the number of apertures and (iii) their position in the grain's surface (panto-, when distributed in homogeneous way, or zono-, when distributed around the equatorial zone of the pollen).

Structure of the cell wall: The two main layers of the cell wall are the inner layer (intine) and the outer layer (exine). Both play an important role for the protection of the male gamete (Lewis 1983). The structure of the exine is variable (e.g. with spines, pores, groves, reticulations over exine), but it is difficult to distinguish the fine details of the different forms. While intine is made of cellulose, exine is made of sporopollenin, which offers resistance to decay. This is an important characteristic for vegetation reconstruction studies (Bolick & Vogel 1992). Sporopollenin is also resistant to chemical reagents (Faegri et al. 1989).

Shape: The shape of pollen grains is spheroidal for most of them or elliptical, oval. To define it, we use the ratio between the length of the polar to the equatorial axis. The observed shape in the optical microscope depends on the polarity (polar or equatorial axis). Pinaceae have a more particular shape, with air sacks, which help their dispersion in the atmosphere (Gottardini et al. 1997).

Size: It ranges from 5 μm (*Myosotis*) to 200 μm (some conifers together with the air sacks), but for the majority of pollen grains it is within the range 20-50 μm. This, in addition to the fact that the size can change because of hydration of the pollen, makes this character not so reliable (Damialis 2010).

With the combination of all these characters, the identification is possible at the family or genus level (Lewis 1983). With additional information, the identification level can reach also to species. The additional information can include botanical data of the area, flowering period in the region or similar geographic regions (Damialis 2010).

The key characteristics for the identification of pollen grains based on their morphological characteristics are grouped in Appendix 1.

Biological processes

The two important biological processes that are related to airborne pollen are the following:

Flowering: It is defined at the individual, population, species or community level (Rathcke & Lacey 1985). Phenology is the study of the timing of periodic biological events (phenophases) like flowering and of their relationship with environmental factors, especially meteorological and climatic ones (Menzel 2000) that are considered important for the changes in the life cycle of organisms (Cleland et al. 2007). The phases of flowering (from bud initiation to end of anthesis) are controlled by the physiological, genetic and ecological responses of each species (Khanduri & Sharma 2000). Due to the increased sensitivity of flowering to environmental changes, phenological data are incorporated as important components in models of biosphere response to climate change (Beaubien & Freeland 2000, Chuine et al. 2000, Menzel et al. 2006).

Pollen production: It is measured per flowering unit (anther, flower or inflorescence). The amount of pollen produced is linked to the pollination mode: it is much lower in the entomophilous species than in the anemophilous (Bhattacharya et al. 1999). The pollen production is determined genetically, but it can vary greatly from one year to another and among sites, due to environmental conditions, the age or the robustness of the individuals (Damialis 2010).

Environmental factors

A number of environmental factors may interact to determine flowering onset, with temperature and light being the main ones in temperate zones (Menzel & Fabian 1999). Several studies have reported that rising spring temperatures, cause advanced flowering (Bradley et al. 1999, Menzel & Fabian 1999, Beaubien & Freeland 2000, Walther et al. 2002). This may be due to the fact that higher temperatures in combination to lower relative humidity can accelerate the matureness of the flowers and the opening of the anthers (Khanduri & Sharma 2000, Jato et al. 2002). Apart from the human-induced climatic changes, biodiversity loss can also cause changes in biotic interactions, which can also indirectly affect flowering (Wolf et al. 2017).

Other factors like the topography (for instance, vicinity to the sea) and altitude can cause differences to the populations' phenological characters (Bradley et al. 1999), as they are associated to spatial variations of the climate (Blionis et al. 2001). For example, it has been found that populations on higher elevations and of south direction flower first, indicating phenotypic plasticity of the flowering characters (Damialis et al. 2010). Studies along an altitudinal gradient can therefore increase our understanding on the plants' phenological responses to thermal regime changes (Jochner et al. 2012).

Changes in the phenological features of flowering may impact many different species, in a wide range of locations. Such changes may be more pronounced at higher latitudes (Bradley et al. 1999). The responses can vary between and within species (Richardson et al. 2017). It is speculated that species without adaptability, i.e. plants in which seasonal development is regulated mainly by photoperiod, or genetic regulatory systems, may experience greater stress or even extinction during extended climate change (Bradley et al. 1999, Richardson et al. 2017). There is also concern about "phenological mismatch" in the mutualistic relationships between flowering plants and their pollinators (Wolf et al. 2017).

Finally, for pollen production similar relationships have been found like for flowering, i.e. meteorological factors, and primarily air temperature, play an important role, with higher temperatures leading to higher production (Damialis et al. 2011).
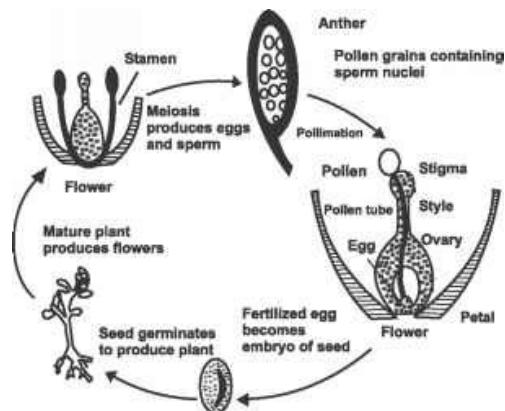
Figure B.1 Life cycle of flowering plants and structure of a flower (from
https://www.medicinalplantsarchive.us)

Σχήμα B.1 Κύκλος ζωής και δομή των ανθοφόρων φυτών (από
https://www.medicinalplantsarchive.us)
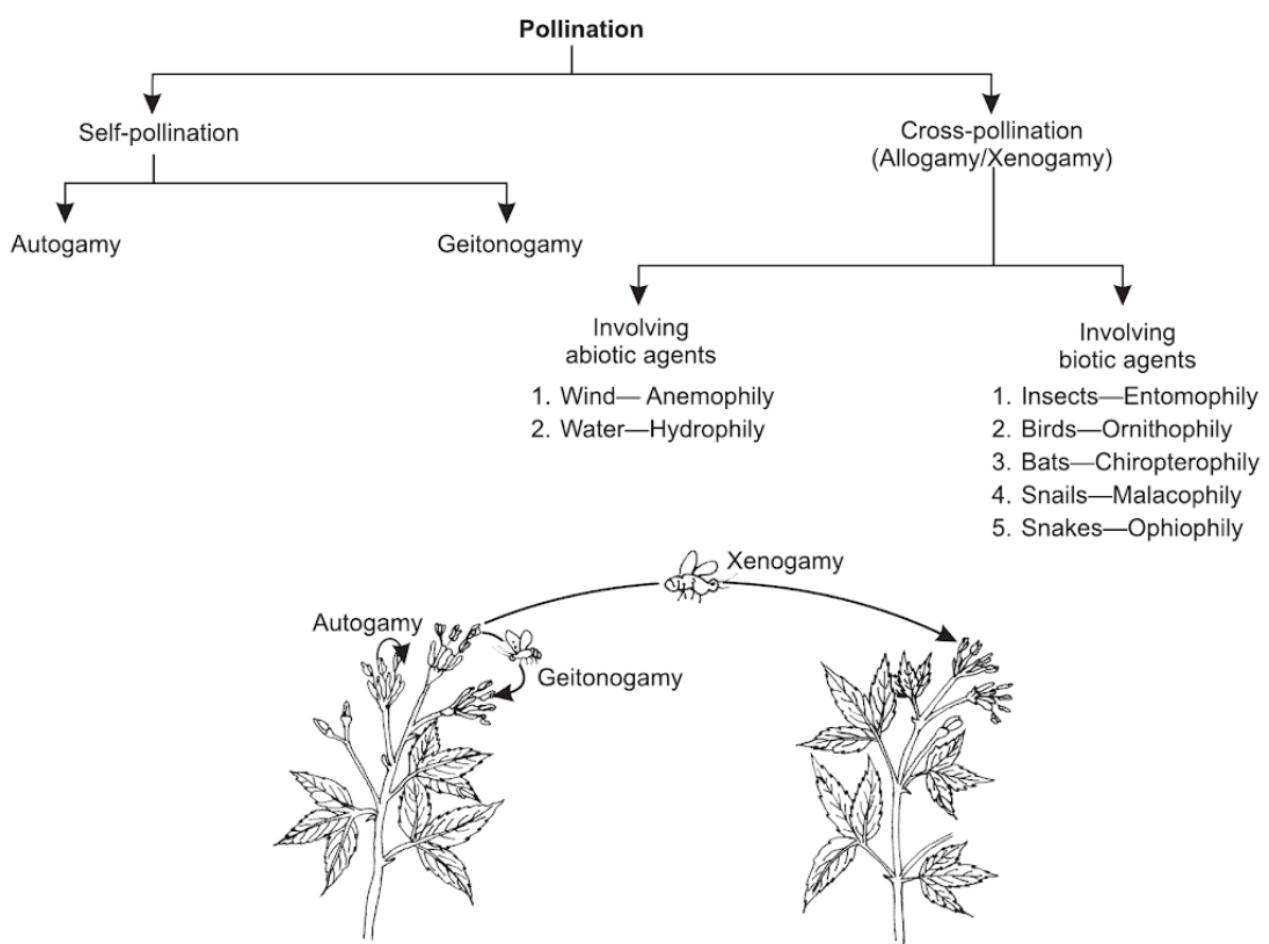

Figure B.2 Pollination types (Venugopal 2016)

Σχήμα B.2 Τύποι επικονίασης (Venugopal 2016)

B.2 Aerobiology

Atmosphere and aerobiological path

The atmosphere consists of gases (mainly nitrogen and also argon and carbon dioxide as trace gases, water vapor at low concentrations) and particulate matter (dust or organic carbon fraction and biological particles) (Flokas 1994). Regarding particulate matter in the atmosphere, it is reported that up to 25% it is comprised of biological particles (Peccia & Hernadez 2006). The airborne biological particles (mentioned in the literature also as 'biological aerosol particles' or 'bioaerosols') can be bacteria, pollen, fungi, viruses, cyanobacteria, microalgae or fragments of them (Despres et al. 2011), generated by the biological activity in the terrestrial and aquatic environments (Mandrioli 1998). They can be transported in the atmosphere primarily with air currents, or vertically, downwards, by gravitational sedimentation, or inside the airborne water droplets and ice crystals. They are removed from the air either by sedimentation on land surfaces, or deposition with precipitation (Despres et al. 2011). The different stages in the movement of airborne particles are presented by the aerobiological path (Lacey and West 2006) (Figure B.3).
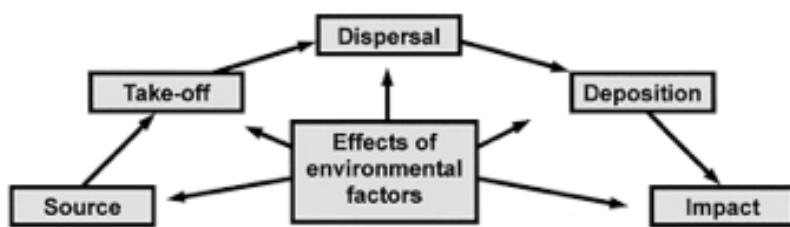
Figure B.3 The path of aerobiology which describes the different stages in the movement of airborne particles (Lacey & West 2006)

Σχήμα B.3 Το μονοπάτι που περιγράφει τα διάφορα στάδια κατά την κίνηση των αερομεταφερόμενων σωματιδίων (Lacey & West 2006)

Field of Research

Aerobiology is a well-established research field that studies airborne biological particles (i.e. their origin, transport and deposition), interacting with other scientific disciplines (e.g. biological, medical, physical) (Frankland 1991). Questions that researchers in this field ask frequently are how, why and when a particle is released into the atmosphere, how it moves, what affects the movement, and what is the impact on the environment and on other organisms (Frenguelli 1998).

The aerobiological records are measurements that inform for the presence and abundance of airborne biological particles in the air; knowing their spectrum we can predict the impact on human health or get useful information regarding environmental processes (Awad 2005, Rodriguez-Rajo et al. 2011).

For airborne pollen, aerobiologists usually study the main characteristics of the pollen season (onset, peak, end, duration) (Damialis et al. 2007), which are useful and find application in different fields:

Public health: Given the pollen allergenicity and the increase in sensitization of the human population, which cause low quality of life and high cost for health care (Reid & Gamble 2009), pollen data can help in the diagnosis and management of allergic diseases, such as respiratory allergy manifested as allergenic rhinitis, allergic bronchial asthma or allergic conjunctivitis (D'Amato et al. 2002).

Ecological and environmental research: Detailed information on the daily, seasonal and annual variation of pollen can lead to long-term predictions of environmental changes (Benninghoff 1991) and climate change impacts (Smith et al 2014). Understanding the relationship between modern pollen deposition and modern plant communities also enables paleoecologists to interpret fossil pollen records and describe plant communities of the past (Hicks 2001).

Other fields: Quite recently a new field of research has been developed, which monitors the spread of genetically engineered pollen from genetically engineered crops in the natural environment (Hofman et al. 2010).

For all the applications mentioned above, there are aerobiological stations continuously monitoring airborne pollen. In Europe, it is estimated that pollen is monitored by a network of about 400 pollen traps (Oteros et al. 2015).

Samplers

Several methods have been developed for air sampling and a wide variety of instruments characterized by different operation and features are manufactured (Levetin et al. 2004). The samplers may have different catchment efficiency, accuracy, degree of automation, simplicity of operation, operation under varying weather conditions, counting and conversion of catch to volumetric basis (Ogden & Raynor 1967). Selection of the sampler may depend on several factors like the needs that they are expected to cover but also existing constraints (e.g. power availability) (West et al. 2015). Summarized below are the principal collection traps for airborne pollen.

Volumetric collectors: These are based on methods that actively sample a certain volume of air and allow calculation of the particles' concentration per cubic meter of air (West et al. 2015). Impaction is the most widely used method, which separates particles from the air stream by using the inertia of the particles (Levetin et al. 2004). The most widely used instrument for monitoring is the Hirst-type trap (Hirst 1952), which is today mainly represented by the Burkard (Burkard Manufacturing, Rickmansworth, Hertsford shire, England) and Lanzoni VPPS 2000 (Lanzoni, Bologna, Italy) devices. The trap is placed most often 10-30 m aboveground. The design of the trap is very simple (http://www.burkardscientific.co.uk): equipped with a vacuum pump, it can draw 10 l of air per minute (mean respiratory capacity of a human) through a thin orifice, which is always oriented to the direction of the wind, thanks to the trap's wind-oriented vane. The particles are impacted on a microscope slide (24-h lid) or a tape (7-day lid) beneath the orifice. The 7-day lid consists of a metal drum, on which an adhesive tape is placed. The drum is clockwork-driven to rotate past the orifice 2 mm per hour, which allows the time-discrimination of the collected particles; in one week a full rotation is completed and the tape is collected. The analytical procedure includes the following: (i) cutting of the tape in

seven equal sections, each representing a day of sampling, (ii) mounting on microscope slides and (iii) conventional analysis that includes identification and counting by use of a light microscope reference identification keys. A subset of the sample is analyzed with at least 10% of the slide read and counts are expressed as number of pollen grains per m^3 of air for each sampling day (Galan et al. 2014). The design of a Hirst-type volumetric sampler is shown in Figure B.4a and B.4b.

Gravimetric collectors: These are non-volumetric sedimentary samplers that rely on gravity in order to assess the composition of airborne material. Gravimetric pollen traps can provide an understanding of pollen transport and deposition (Faegri & Iversen 1989) and they have been used efficiently in remote areas, like forests where they have been proved to be representative of the local vegetation (Haselhorst et al. 2013). Tauber traps (Tauber 1967) were established as modified 'Tauber traps' from the European Pollen Monitoring Programme (1996) and they have been used by paleoecologists to study the pollen deposition (influx) of certain species so as to interpret how this is related to vegetation and climatic changes (Hicks et al. 2001, Kvavadze 2001). Tauber traps are cylindric plastic containers, filled with a conservative substance. Without the need of electric supply, they can be left in the field for long periods. Pollen is captured in a liquid and later on a filter, from which the created pellet can be used for direct analysis. It is considered one of the most inexpensive and simple methods, since the requirements are very low (Levetin 2004). However, the air volume sampled is unknown and the counts are expressed per surface (number of pollen grains per m^2). The design of Tauber trap is shown in Figure B.4c.
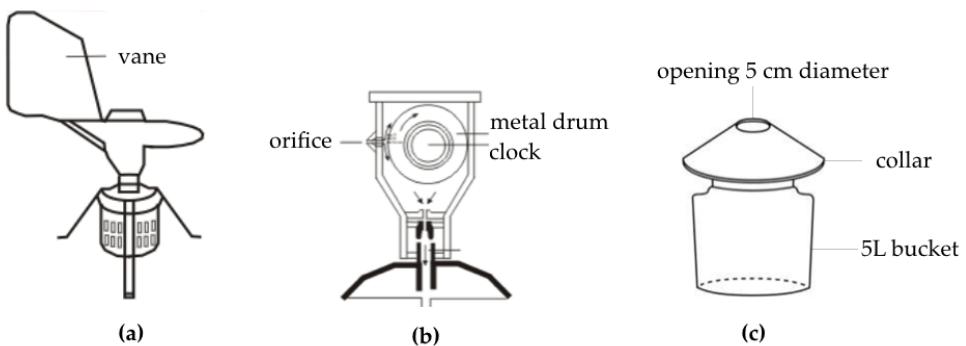
Figure B.4 (a) Hirst-type volumetric trap **(b)** Hirst-type volumetric trap in cross-section and **(c)** Gravimetric Tauber trap

Σχήμα B.4 (a) Ογκομετρική *παγίδα τύπου* Hirst **(b)** Ογκομετρική *παγίδα τύπου* Hirst *σε διατομή και* **(c)** *Παγίδα καθίζησης τύπου* Tauber

Influencing factors

The airborne pollen patterns can be influenced from all the factors affecting the two pollen related biological processes (described in B.1). Other factors that affect directly the airborne pollen patterns are discussed below.

Vegetation of the area: Airborne pollen reflects local and regional sources (Charalampopoulos et al. 2013). However, the relationship between pollen occurrence and vegetation composition is not 1:1, since there can be differences in pollen production, pollen dispersal and pollen deposition between species (Hicks 2006). For example, the time period that pollen remains in the air and the settling velocities may depend on the species-specific shape and size of the pollen (Despres et al. 2011), while the quantities produced depend on the pollination mode of the plant (Appendix 4). Airborne pollen in an area is mainly from anemophilous and to a smaller extent from entomophilous plants (Despres et al. 2011, Jørgersen et al. 2012). Finally, pollen may arrive from long-distance sources (Van de Water 2003, Damialis et al. 2016, Mohanty et al. 2017).

Time of the year: Pollen presence in the atmosphere follows a clear seasonal cycle in response to the flowering seasons of the plant sources (Despres et al. 2011). Most of the plants flower in spring, but some of them start very early, already in January, or very late

in summer or autumn (Appendix 4).

Abiotic factors: Release and dispersal of pollen depends on meteorological factors. In particular, the bonding of pollen to surfaces is influenced by increases in air temperature and decreases in humidity (Jones & Harrison 2004). Pollen emission is reduced in the presence of rain or when the wind speed is low (Damialis et al. 2005, Despres et al. 2011). Dispersal depends also on re-suspension, when sedimented pollen from dry surfaces becomes again airborne (Despres et al. 2011). It has been found also that pollen concentrations decrease with altitude (Despres et al. 2011, Charalampopoulos et al. 2013). However, in mountainous areas, the variability is high, due to the very variable environmental factors (air temperature, precipitation, relative humidity, wind speed and direction) and the physical form of the mountains (direction, altitude) that influence the way the mountain acts, i.e. as a source or as an obstacle for pollen transport (Charalampopoulos et al. 2013). In particular, Jochner et al. (2012) found the highest and vegetation-free sites to be affected by turbulent vertical mixing and wind transport of pollen.

Other factors: The type of sampler and the sampling techniques could also introduce variations (Pedersen & Moseholm 1993, Levetin et al. 2000).

B.3 Methods for pollen analysis

Samples collected from the samplers described above can be analyzed by various methods, based on the type of the sample and the information desired (Levetin 2004). The main pollen analysis methods are described below and shown in Figure B.5.

Microscopy: Light microscopy is the most common method of pollen identification based on pollen morphological features (Despres et al. 2011). Here, well-known limitations are (i) low taxonomic resolution, since only a small percentage of pollen grains are identifiable to genus and species level, due to common morphological features shared within genera,

families and even orders, (ii) low time efficiency, especially in large-scale studies, since considerable time is required for pollen identification and enumeration and (iii) low data reproducibility, since the identification may be subjective, depending on the researcher (Despres et al. 2011). Other types of microscopy include (i) scanning electron microscopy (SEM), which delivers high-level classification results, but requires expensive microscope equipment and specific sample preparations (Dell' Anna et al. 2010), (ii) fluorescence microscopy (Pohlker et al. 2011), which takes advantage of the autofluorescence signatures of different pollen types, but is tedious when particles get classified and counted by people (Despres et al. 2011).

Spectroscopic techniques: They correspond to biochemical characterization of pollen grains, based on absorption and scattering fingerprints (Dell' Anna et al. 2010). By use of Fourier Transform Infrared Spectometer (FTIR), species belonging to the same genus can be distinguished (Pappas et al. 2003, Gottardini et al. 2007). However, for application in aerobiological monitoring networks the spectra acquisition is a time-consuming step (Dell' Anna et al. 2010).

Automatic recognition: It provides optical detection of pollen in real-time and with high resolution, based on machine learning and supervised classification and it reduces the need for experts during the analysis (Dell' Anna et al. 2010). It is claimed that a finer temporal resolution of aerobiological data allows better correlation with meteorological data, especially wind data, hence, a better interpretation and understanding of aerobiological processes (Jochner et al. 2012). It would be also more effective for allergy sufferers (Crouzy et al. 2016). Automatic recognition of pollen grains has been applied with the use of microscopic images and computer image analysis (Bonton et al. 2002). The most recent pollen monitoring system that is based on image recognition is the BA500, a 3-stage impactor equipped with microscope and a camera (Oteros et al. 2015, www.hund.de/en/instruments/pollen-monitor), and the cytometer Rapid-E Plair based on scattering and fluorescence (Crouzy et al. 2016, http://www.plair.ch).

DNA-based methods: Molecular identification at different taxonomic levels can be

achieved with PCR-based approaches that use a wide range of DNA markers of the three plant genomes (nuclear, mitochondrial and chloroplast) (Georgolopoulos et al. 2016). DNA-based airborne pollen analysis has been already applied to aerobiological research, using the classical volumetric Hirst-type sampler (Longhi et al. 2009, Kraaijeveld et al. 2015, Ghitarrini et al. 2018). Longhi et al. (2009) used real-time PCR techniques and taxon-specific primers for pollen identification. They obtained promising results even with pollen mixtures and argued that a simultaneous identification of different taxa would reduce the time and effort of the analysis. Ghitarrini et al. (2018) followed the same approach to discriminate Poaceae taxa in aerobiological samples. Quantitative analysis by PCR was applied also to detect genetically modified pollen (Hofman et al. 2010) and for analysis of floral origin in honey (Guertler et al. 2013). Other authors used PCR, cloning and Sanger sequencing for the same applications (Wilson et al. 2010, Folloni et al. 2012, Galimberti et al. 2014, Bruni et al. 2015). Environmental samples from different sources can be now used to detect biodiversity via environmental DNA and next generation sequencing, with several applications, such as to detect allergens in the air (Kraaijeveld et al. 2015, Núñez et al. 2017), to study plant-pollinator interactions (Pornon et al. 2016, Bell et al. 2017), detect the provenance of honey (Valentini et al. 2010, Hawkins et al. 2015, Richardson et al. 2015a) or reconstruct past vegetation (Jørgensen et al. 2012); much less research focuses on contemporary plant communities. Kraaijeveld et al. (2015) proposed NGS as a successful method not only to identify multiple pollen taxa simultaneously, but also to distinguish grass-allergen genera, and concluded that the method could easily be applied to other bioaerosols.
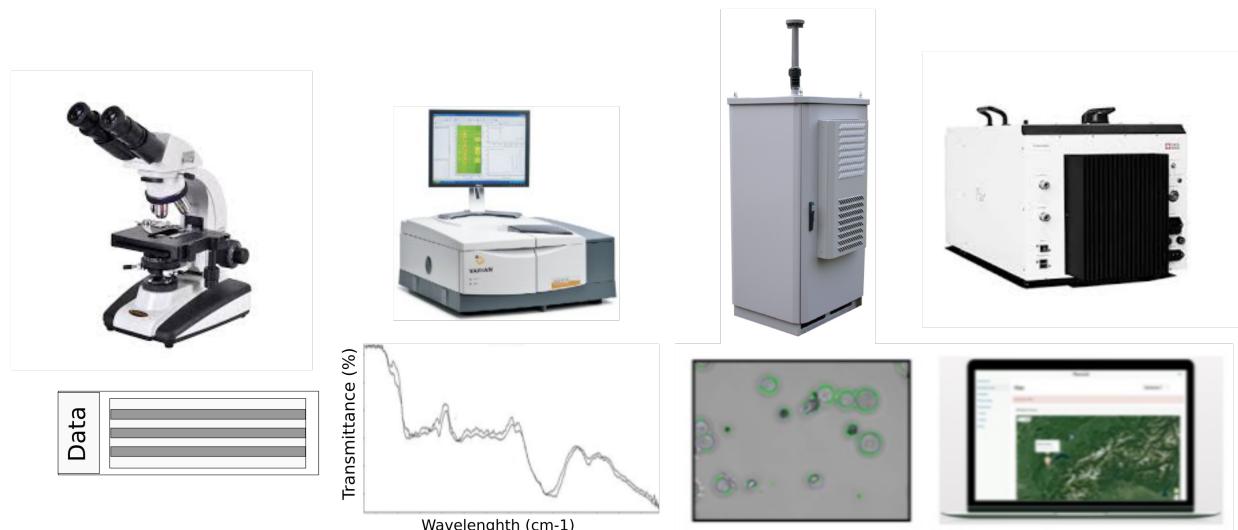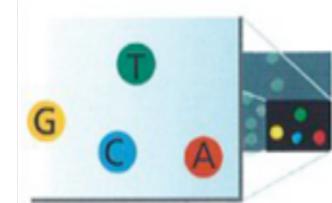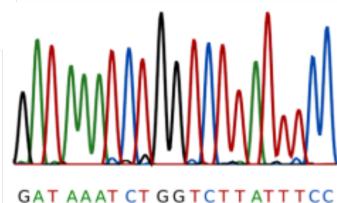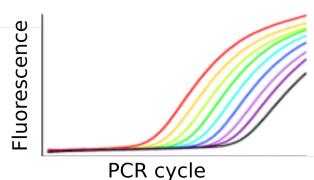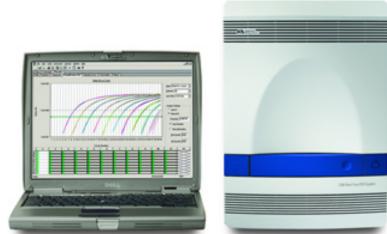
Figure B.5 Pollen analysis methods used in aerobiology

Σχήμα B.5 Μέθοδοι ανάλυσης γύρης που έχουν χρησιμοποιηθεί στην αεροβιολογία

B.4 Environmental DNA: definitions and analysis

Definitions

Environmental DNA (eDNA): DNA isolated in traces from an environmental sample, without previous isolation of any target organism (Taberlet et al. 2012). Traces of DNA can originate from feces, mucus, skin cells, organelles, gametes or even extracellular DNA (Deiner et al. 2017). The environmental samples may have their source in modern (e.g., seawater, freshwater, soil or air) or ancient environments (e.g., cores from sediment, ice or permafrost) (Deiner et al. 2017).

Barcoding: taxonomic identification of a single species using a diagnostic marker (Hebert et al. 2003). The principle of the method is that a specific fragment of DNA (marker) is targeted, which is variable enough to allow taxonomic identification. For plant-barcoding studies, plastid (e.g. the protein coding barcodes rbcL and matK, and the non-coding spacer trnH-psbA) and nuclear regions (e.g. the internal transcribed spacer of the ribosomal DNA) have been used (Hollingsworth et al. 2016).

Metabarcoding: taxonomic identification of multiple species extracted from a mixed sample. Here a specific fragment of DNA is targeted, which is similar enough across taxa to be amplified using the same primers (universal primers), yet enough variable to allow taxonomic identification. Plant studies focusing on mixed templates and/or degraded DNAs (e.g. environmental samples) typically use the P6 loop of the plastid trnL intron, whose short length and conserved primer sequences make it particularly suitable for amplification and next generation sequencing technologies (Hollingsworth et al. 2016).

Operational Taxonomic Unit (OTU): taxonomic group defined by the researcher in a study (e.g. individuals, populations, species, genera, or strains) with the use of clustering according to a predefined sequence similarity, as described by Blaxter et al. (2005).

Sanger sequencing: DNA sequencing method (i.e. process to determine the exact order of nucleotides within a DNA molecule), based on the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase. In particular, chain terminating

dNTPs are incorporated, which are missing the 3-OH group required for the phosphodiester bond between two nucleotides, DNA polymerase stops the extension of DNA and DNA fragments are separated by size in capillary electrophoresis. This technique uses laser to activate the fluorescent dideoxy nucleotides and a detector to distinguish the colours, represented by a chromatogram. The method may have provided good quality results, but has a read length limitation. Also, to sequence millions of base pairs is time-consuming and expensive. In 1986, Applied Biosystems commercialized a fluorescent DNA sequencing instrument, almost the same period that PCR was proposed by Kary Mullis for the amplification of DNA sequences and for two decades this is the primary method for sequencing (Mardis 2013).

Next generation sequencing (NGS) (or second-generation sequencing, or High Throughput Sequencing, HTS): From 2005 on, new instrumentation introduced the concept of next generation sequencing, with the main difference that here millions of sequence reads can be processed in parallel (Mardis 2008). The workflow starts with random fragmentation of DNA into a library of small segments and preparation for sequencing by ligating adaptor oligonucleotides to both ends of each DNA fragment (Mardis 2008). Other features and principles differ among the different NGS instruments (Mardis 2008, Kchouk et al. 2017) (Table B.1).

eDNA biodiversity studies

Monitoring biodiversity in natural ecosystems and particularly in protected areas (e.g. the European network NATURA 2000) is becoming of increasing importance in ecological research (Charalampopoulos et al. 2013). With recent developments of DNA metabarcoding, a powerful tool is available to survey rapidly plant and animal communities and provide more detailed information about biodiversity changes over long time periods and large regions (Deiner 2017). More interestingly, the obtained molecular data give now new insights on membership in groups of organisms and on alpha and beta diversity of different communities and ecosystems, possibly never studied before (Creer et

al. 2016). With such deeper knowledge in basic research, important ecological issues can be addressed more efficiently, such as human-induced change of climate, land use or other issues like pollution, habitat loss and introduction of invasive species (Yoccoz et al. 2012).

Biodiversity studies have been conducted with environmental samples from aerial, terrestrial, freshwater and marine ecosystems (Appendix 2) with applications in the fields of microbial ecology (Zinger et al. 2009, Barberàn et al. 2012, Blaalid et al. 2012, Stoof-Leichsenring et al. 2012, Bruno et al. 2017), diversity of plants and animals, or diet analysis (Pompanon et al. 2012, Shehzad et al. 2012, De Barba et al. 2014).

There have been several methodological and technical advances (Coissac et al. 2012, Epp et al. 2012, Shokralla et al. 2012, Taberlet et al. 2012, Boessenkool et al. 2014) related to metabarcoding and NGS, which make the production of DNA sequences fast and easy (Schokralla et al. 2012). However, the molecular identification system still needs improvement for specific fields of study, since the current surveys do not always agree on the consistency of the obtained information to that from traditional approaches (Bienert et al. 2012, Yoccoz et al. 2012, Calvignac et al. 2013, Kraaijeveld et al. 2015, Núñez et al. 2017, Richardson et al. 2015a). Especially for plants, this has to do with the discriminatory power of the selected marker and the bioinformatics approaches used in the analysis (Sandionigi et al. 2012).

Methodological aspects

A metabarcoding approach commonly includes several important methodological steps that should be taken into consideration when designing workflows (Murray et al. 2015).

DNA extraction: Cell lysis and DNA purification need to be customized for the type of sample examined (Bell et al. 2016). Especially pollen is a challenging material for the extraction of DNA because the cells are surrounded by the resilient cellulose-rich intine and the sporopollenin-containing exine (Kraaijeveld et al. 2015). There are several studies examining how the disruption methods and methods using phenol-chlorophorm or the commercial kits may influence the DNA yield and quality, suitability for downstream PCR

amplifications or influence diversity indices (Dineen et al. 2010, Henderson et al. 2013, Lazarevic et al. 2013, Fliegerova et al. 2014, Kennedy et al. 2014).

PCR amplification: The appropriate conditions of PCR variables are studied to obtain good quality of amplified fragments. These variables are number of PCR cycles (Wu et al. 2010, Ahn et al. 2012), DNA polymerase type (Arezi et al. 2003, Wu et al. 2010), primers (Ihrmark et al. 2012) and amplicon size (Hubet et al 2009, Engelbrektson et al. 2010). Chimera formation (Stevens et al. 2013), primer mismatch (Sipos et al. 2006) and other PCR biases (Cai et al. 2013) are issues that may occur. Template dilution (Wu et al. 2010), replication (Ficetola et al. 2015) and *in silico* analyses are proposed for selection of markers and primers (Bellemain et al. 2010).

Sequencing: Since the existing sequencing platforms have different inherent technical limitations (Luo et al. 2012, Quail et al. 2012, Werner et al. 2012, Fadrosh et al. 2014), sequence depth (Smith et al. 2014) and library preparation protocols (Dijk et al. 2014, Head et al. 2015), the diversity captured by each should be considered.

Analytical approach: A comprehensive reference database from verified barcode sequences of the main taxa in the research area is necessary for taxonomic identification. If not available, it needs to be created (Taberlet et al. 2007). Bioinformatics challenges are related with reduction of the noise of sequencing data (Quince et al. 2009, Kunin et al. 2010, Quince et al. 2011, Bakker et al. 2012, Rosen et al. 2012, Edgar 2013, Gaspar et al. 2013, Morgan et al. 2013), selection of the OTU clustering method (Doolittle et al. 2006, Huse et al. 2010, Blaalid et al. 2013, Hwang et al. 2013, Patin et al. 2013, Preheim et al. 2013, Lekberg et al. 2014), detection of chimeric sequences (Wang & Wang 1996, Haas et al. 2011, Fonseca et al. 2012), identification of rare taxa (Elshahed et al. 2008, Biesbroek et al. 2012), taxonomic annotation issues (Nilsson et al. 2006, Koljalg et al. 2013) and level of taxonomic resolution (Olsgard 1998, Schmidt-Kloiber & Nijboer 2004). Finally, quantitative issues between the copy number of genes and the the diversity and abundance are also investigated (Kembel et al. 2012, Darby et al. 2013).

Other issues: The effect of sampling and storage conditions (Prosser et al. 2010, Taberlet et

al. 2012, Vickova et al. 2012, Fliegerova et al. 2014) on the extracted DNA and the reproducibility of the method applied (Zhou et al. 2011) have been also addressed.

Table B.1 Next (or second) generation sequencers (Mardis 2008, Kchouk et al. 2017)

Πίνακας B.1 Όργανα για αλληλούχιση νέας (ή δεύτερης) γενιάς (Mardis 2008, Kchouk et al. 2017)

| Insrument	Year	Principal	Output
Roche (454) GS FLX sequencer or pyrosequencing (http://www.454.com)	2004	Releases pyrophosphate molecule when DNA polymerase incorporates nucleotides and uses emulsion PCR to amplify DNA on a surface of agarose beads attached with oligomers, each of which is complementary to the adaptor sequences that were ligated to the fragment ends during library construction	Programmed to sequence 24 96-well plates per day, produces 440 kb of sequence data in 7 hours, with an average read length of 650 bp per sample.
Illumina genome analyzer (http://www.illumina.com)	2006	Uses a flowcell of eight lanes (each one attached with oligos complementary to the specific adapters that are ligated onto the library fragments), suitable for bridge amplification, an isothermal process that amplifies each fragment into a cluster, which is then denatured, annealed with a sequencing primer and subjected to 'sequencing by synthesis' using 3' blocked labeled nucleotides	At the end of the sequencing run (4 days), a typical run yields 40–50 million sequences. Since 2011, MiSeq can produces 15 GB of data, with average read length 300 bp.
Applied Biosystems SOLiD sequencer (http://www.lifetechnologies.com)	2007	Attaches adaptors to DNA fixed with magnetic beads and amplifies each bead–DNA complex by emulsion PCR. Sequencing by ligation first anneals a universal sequencing primer, then goes through subsequent ligation of the appropriate labeled 8mer, followed by detection at each cycle	Requires 5 days and produces 3–4 Gb of sequence data with an average read length of 25–35 bp.
Ion Torrent (https://www.thermofisher.com/us/en/home/brands/ion-torrent.html)	2010	Based on the detection of the hydrogen ion released during the sequencing process. This change is detected by a sensor attached to the bottom of the micro well and converted into a voltage signal which is proportional to the number of nucleotides incorporated.	Capable of producing longer read lengths of 200 bp, 400 bp and 600 bp with throughput that can reach 10 Gb. Fast sequencing time between 2 and 8 hours.

Measures of biodiversity

Biodiversity measures take into account incidence data or incidence and abundance data. However, when dealing with DNA data, the use of abundance data is not always reliable, due to variations in gene copy number among taxa and PCR biases (Zinger et al. 2012). Biodiversity is expressed by alpha, beta and gamma diversity (Whittaker 1972) as described below and shown in Figure B.6.

Alpha diversity: The diversity within a site or a sample, representing the community's richness in species (species richness) or the extent of species dominance (species evenness).

Beta diversity: The differentiation of communities along habitat gradients and hence between two or more sites or samples. To measure beta diversity there are two main approaches considering (i) incidence data, with metrics such as of Jaccard and Sorensen, and (ii) relative abundance data, with metrics such as of Bray-Curtis.

Gamma diversity: the total diversity of a landscape or geographic area, estimated after alpha and beta diversities.
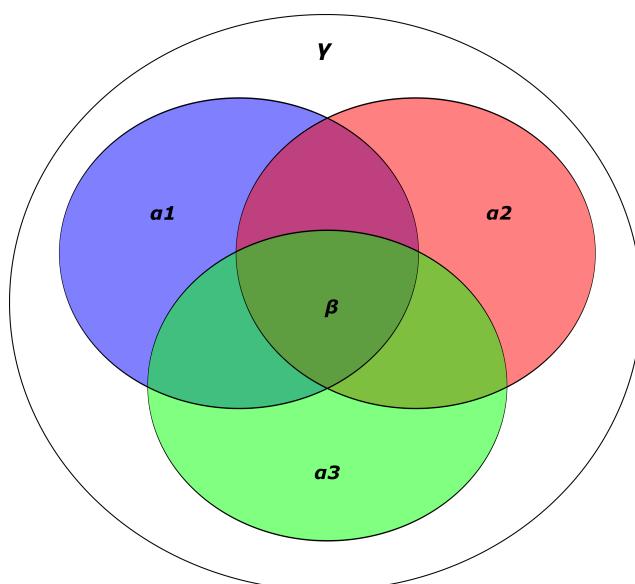
Figure B.6 Illustration of alpha diversity (α) of three communities, of beta (β) diversity between them and of gamma (γ) diversity of the whole region where these communities belong

Σχήμα B.6 Άλφα (α) ποικιλότητα τριών βιοκοινοτήτων, βήτα (β) ποικιλότητα μεταξύ αυτών και γάμα (γ) ποικιλότητα ολόκληρης της περιοχής στην οποία εντάσσονται.

Chapter C. Study area

C.1 Vegetation

The study area is Paneveggio - Pale di San Martino national park, located in eastern Trentino, province of northern Italy (46°18′28″N 11°44′38″E). It is mainly a mountainous area covering a total surface of almost 20,000 hectares. More than 90% of the park lies above 1500 m, within the range 1500-2500 m (Amadei et al. 2005). It is characterized by a diversity of habitat types and a rich flora, of 1451 plant species (https://www. parcopan.org). The main feature of the eastern part of Paneveggio is the prevalence of dolomitic limestone substrate with screes and bare rocks of chasmophytic vegetation. In this part, there are the three highest peaks of the Park (max elevation 3192 m). The forested zones of the area consist of *Fagus sylvatica* and *Abies alba*, from 1200 to 1550 m, and of *Picea abies* (spruce), from 1550 to 1850 m, further distinguished in mountainous spruce forests below 1600 m and subalpine spruce forests above. *Larix decidua* and *Pinus cembra* participate in the forests of higher altitudes, between 1800 and 2100 m. *Pinus mugo* and *Rhododendron* participate in the subalpine vegetation. Meadows and heaths are common habitat types in the alpine and subalpine areas. The conifer forests cover in total 47.6% of the area, while hardwood forests only 1.1% (Amadei et al. 2005). The dominant tree of the park (85%) is *Picea abies* (Piano del Parco, Parco Naturale Paneveggio Pale di San Martino). All the habitats present in the Park according to CORINE biotopes are presented in Figure C.1. The habitats according to EU NATURA 2000 classification, are presented in Table C.1. From the lowest to the highest elevations, the important habitats and phytosociological units encountered (Amadei et al. 2005) are the following:

Illyrian *Fagus sylvatica* forests: This forest type represents the thermophilous Aremono-Fagion community, with dominant species the beech, *Fagus sylvatica.* It is distributed at low elevations in calcareous and dolomitic substrates. In the park, it is present in the south (Val Cismon and Val Canali), where it finds more favorable climatic conditions. In

Paneveggio, there is also the category of medio-European beech forests, with the Cephalanthero-Fagion community. In this, there are some thermophilous species like *Primula vulgaris*. Mixed woodlands of *Fagus sylvatica* with *Ostrya carpinifolia* can also be included here.

Acidophilous *Picea* forests of the montane to alpine levels: It is the most widespread forest type in the park. In this forest type, the main phytosociological group is the Vaccinio-Piceetea in substrates both calcareous and siliceous. Spruce often gets mixed with larch towards the upper boundaries of the formation. Homogyno-Piceetum is found in the acidic substrates and Adenostyle glabrae-Piceetum in the carbonate substrates. The montane spruce forests include also the calcific association Calamagrostio variae-Piceetum and the acidophilus one Soldanello montanae-Piceetum.

Alpine *Larix decidua* and/or *Pinus cembra* forests: The two species may form either pure or mixed forests and may be associated with *Picea abies*. The biggest part of the park is Larici cembretum, while there is also Laricetum and Cembretum. In the park, they are mainly present in the northernmost part, above the subalpine *Picea*-forest.

Alpine and boreal heaths: They are mainly represented by the Junipero-Rodoreto community, which is found in both acidic and calcareous substrates, and includes shrub formations (secondarily there is also *Pinus mugo* and *Alnus viridis*). Dominant acidophilous communities are the Rhododendro-Vaccinion (Vaccinio-Piceetea), while those in the calcareous substrates are related to Erico-Pinion mugo and Ericion carneae.
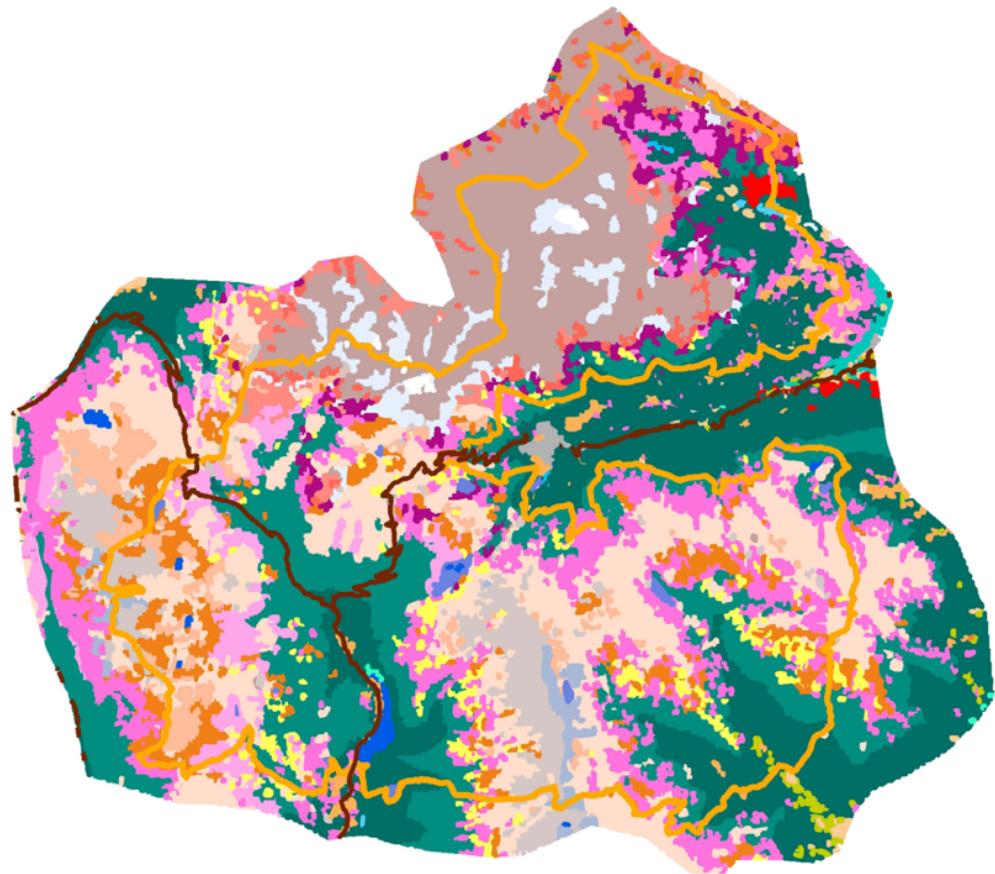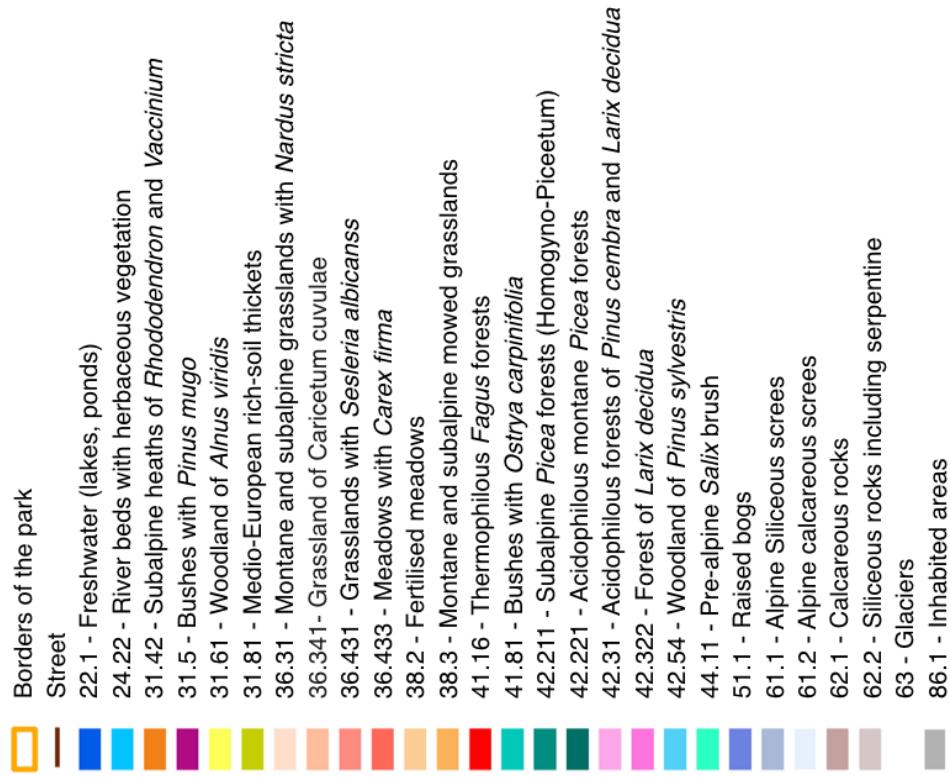
Legend

Borders of the park

Street

22.1 - Freshwater (lakes, ponds)
24.22 - River beds with herbaceous vegetation
31.42 - Subalpine heaths of *Rhododendron* and *Vaccinium*
31.5 - Bushes with *Pinus mugo*
31.61 - Woodland of *Alnus viridis*
31.81 - Medio-European rich-soil thickets
36.31 - Montane and subalpine grasslands with *Nardus stricta*
36.341- Grassland of Caricetum cuvulae
36.431 - Grasslands with *Sesleria albicanss*
36.433 - Meadows with *Carex firma*
38.2 - Fertilised meadows
38.3 - Montane and subalpine mowed grasslands
41.16 - Thermophilous *Fagus* forests
41.81 - Bushes with *Ostrya carpinifolia*
42.211 - Subalpine *Picea* forests (Homogyno-Piceetum)
42.221 - Acidophilous montane *Picea* forests
42.31 - Acidophilous forests of *Pinus cembra* and *Larix decidua*
42.322 - Forest of *Larix decidua*
42.54 - Woodland of *Pinus sylvestris*
44.11 - Pre-alpine *Salix* brush
51.1 - Raised bogs
61.1 - Alpine Siliceous screes
61.2 - Alpine calcareous screes
62.1 - Calcareous rocks
62.2 - Siliceous rocks including serpentine
63 - Glaciers
86.1 - Inhabited areas

Figure C.1 Map of habitat types in Paneveggio National Park based on CORINE land use (Amadei et al. 2005)

Σχήμα C.1 Χάρτης με τους τύπους οικοτόπων στο Εθνικό πάρκο Paneveggio με βάση τη χρήση γης του CORINE (Amadei et al. 2005)

41

Table C.1 Habitat types in Paneveggio-Pale di San Martino National Park according to the EU coding system.

Πίνακας C.1 Τύποι οικοτόπων του Εθνικού πάρκου Paneveggio-Pale di San Martino, σύμφωνα με το σύστημα κωδικοποίησης της Ευρωπαϊκής Ένωσης.

| Paneveggio habitats	Habitat code	Cover (%)
Acidophilous *Picea* forests of the montane to alpine levels	9410	24.31
Siliceous alpine and boreal grasslands	6150	13.77
Alpine *Larix decidua* and/or *Pinus cembra* forests	9420	12.82
Alpine and boreal heaths Rodoreto acidofilo, Junipero-Rodoreto	4060	10.92
Calcareous rocky slopes with chasmophytic vegetation	8210	9.20
mix of diverse land cover not categorized	non-EU	8.64
Calcareous and calcshist screes of the montane to alpine levels	8120	4.99
Illyrian *Fagus sylvatica* forests	91K0	3.62
Alpine and subalpine calcareous grasslands	6170	2.44
Limestone pavements	8240	2.44
Bushes with *Pinus mugo* and *Rhododendron hirsutum*	4070	2.19
Siliceous scree of the montane to snow levels	8110	1.84
Siliceous rocky slopes with chasmophytic vegetation	8220	1.27
Mountain hay meadows	6520	0.33
Transition mires and quaking bogs	7140	0.29
Permanent glaciers	8340	0.28
Alkaline fens	7230	0.15
Species-rich *Nardus* grasslands, on siliceous substrates in mountain areas (and submountain areas in Continental Europe)	6230	0.11
Alpine rivers and the herbaceous vegetation along their banks	3220	0.09
Alluvial forests with *Alnus glutinosa* and *Fraxinus excelsior*	91E0	0.08
Oligotrophic to mesotrophic base poor standing waters of planar to subalpine zones of the Continental and Alpine regions and mountain ranges	3130	0.05
Alpine rivers and their ligneous vegetation with *Salix elaeagnos*	3240	0.04
Natural eutrophic lakes with Magnopotamion or Hydrocharition-type vegetation	3150	0.04
Semi-natural dry grasslands and scrubland facies on calcareous substrates	6210	0.04
Bog Woodland	91D0	0.02
Luzulo-Fagetum beech forests	9110	0.02
Alpine pioneer formations of the Caricion	7240	0.01

C.2 Selection of sampling sites

The sampling sites are located on San Martino chain (Tognola mountain), in the west and the southeastern chain of Val Canali. For the sampling design, we used a map of the Park (downloaded from www.OpenDataHub.it), on which we defined sampling sites of 1x1 km, all of eastern exposition, corresponding to different habitats types, both within and outside the EU NATURA 2000 local network. Within each site, three random points, on which to sample, were selected (Figure C.2).

After the EU NATURA 2000 classification, there are 29 habitat types in the Park comprising 91.4% of its area; another 8.6% is covered by non-EU habitats. We chose four of the most represented habitats on the mountain, in terms of the area that they cover: Acidophilous *Picea* forests of the montane to alpine levels (24.31%), Alpine *Larix decidua* and/or *Pinus cembra* forests (12.82%), Alpine and boreal heaths (10.92%) and Illyrian *Fagus sylvatica* forests (3.62%). The *Picea*-forest habitat was studied at two different altitudinal ranges. At low altitudes, two open areas and a forested one, all outside the Park, were also selected for study (Table C.2). From now on, they will be referred to as 'lowland' (although typically they are not).

Table C.2 The habitat types (according to the EU coding system) that were selected for the study. Given in parentheses are the abbreviated names of the habitat types that we use in the text and figures.

Πίνακας **C.2** Τύποι οικοτόπων (σύμφωνα με το EU σύστημα κωδικοποίησης) που επιλέχθηκαν για μελέτη. Σε παρένθεση δίνονται με συντομογραφία τα ονόματα των οικοτόπων που χρησιμοποιούμε στο κείμενο και στα σχήματα.

Habitat type	Altitude (m)	Sampling area
Lowland	1050-1080	Val Canali
Illyrian *Fagus sylvatica* forests (*Fagus* habitat)	1290-1460	Val Canali
Acidophilous *Picea* forests of the montane to alpine levels (*Picea* habitat-low)	1530-1590	Val Canali
Acidophilous *Picea* forests of the montane to alpine levels (*Picea* habitat-high)	1620-1760	Tognola
Alpine *Larix decidua* and/or *Pinus cembra* forests (*Larix* habitat)	1780-1860	Tognola
Alpine and boreal heaths (Alpine)	2040-2180	Tognola
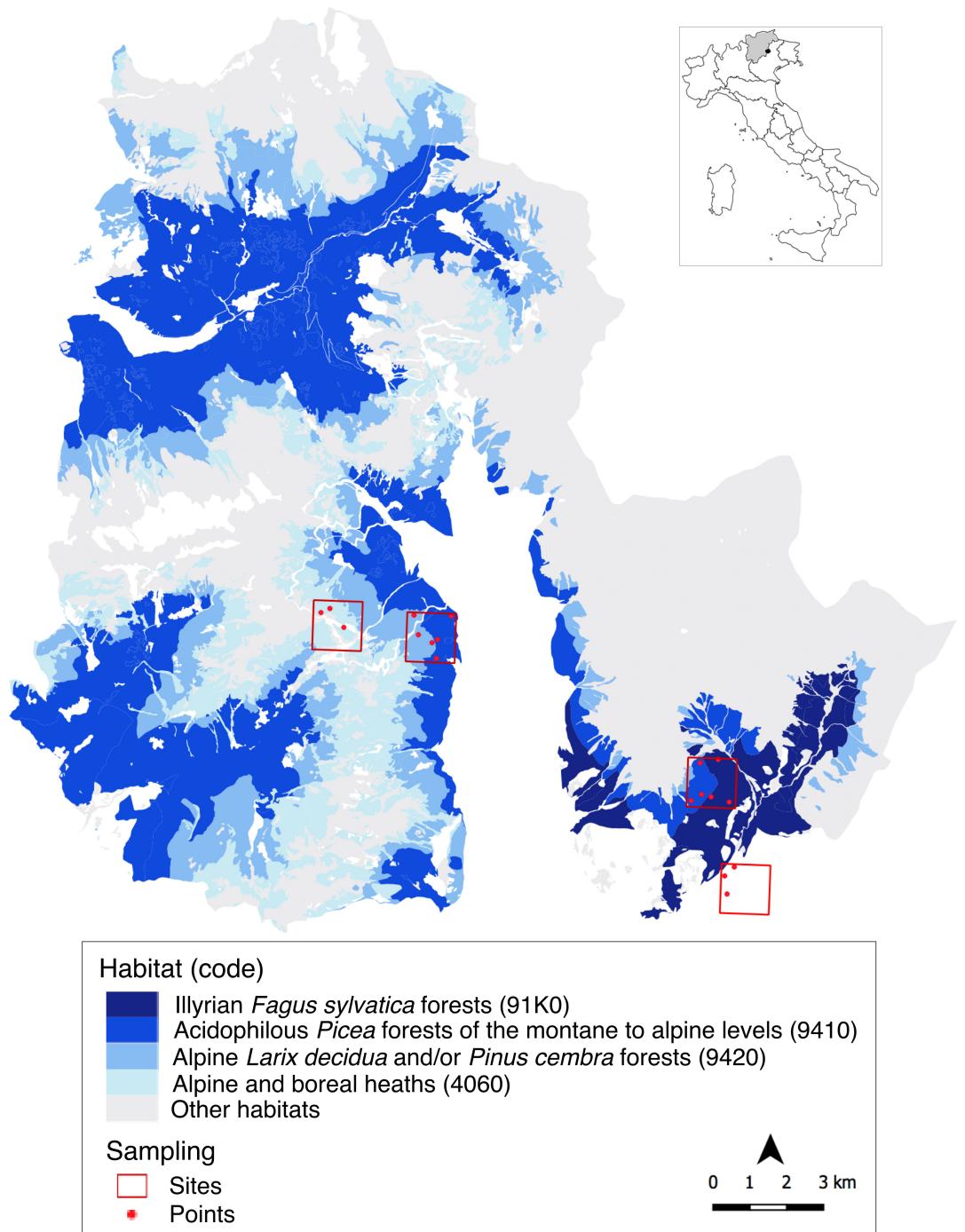
Figure C.2 The sampling area in the National Park of Paneveggio – Pale di San Martino, Italy. Marked are the habitat types that were selected for study, the sampling sites and the sampling points.

Σχήμα C.2 Περιοχή δειγματοληψίας στο Εθνικό πάρκο Paneveggio – Pale di San Martino, Ιταλία. Σημειώνονται οι τύποι οικοτόπων που επιλέχθηκαν προς μελέτη καθώς και οι περιοχές και τα σημεία δειγματοληψίας.

C.3 Tauber traps

Gravimetric Tauber traps were positioned at 18 points within these habitat types. At each sampling point there were two replicate traps: one for microscopic analysis and one for molecular analysis (Figure C.3). There were three sampling periods: October 2014-March 2015, March-July 2015 and July-October 2015.

Each Tauber trap had 700 mL of a preservative solution (1:1:1 water, alcohol, glycerol, plus 2 g l^{-1} phenol). All traps were put close to the ground on poles that were manufactured and positioned at the different sampling points. A collar was placed on their apertures and the airborne particles were collected by gravitation settling. The aperture was covered with a 5 mm mesh net to prevent collection of larger particles.

Figure C.3 Tauber traps positioned in different habitats of Paneveggio National Park 1: Lowland, 2: Illyrian *Fagus sylvatica* forests, 3: Acidophilous *Picea* forests of montane to alpine levels, 4: Alpine *Larix decidua* and/or *Pinus cembra* forests, 5: Alpine and boreal heaths

Σχήμα C.3 Παγίδες τύπου Tauber τοποθετημένες σε διαφορετικούς οικοτόπους του Εθνικού πάρκου Paneveggio-Pale di San Martino 1: πρόποδες, 2: Illyrian *Fagus sylvatica* forests, 3: Acidophilous *Picea* forests of montane to alpine levels, 4: Alpine *Larix decidua* and/or *Pinus cembra* forests, 5: Alpine and boreal heaths

Chapter D. Local reference database

A first important step for a metabarcoding approach is that a comprehensive reference database needs to be created from verified barcode sequences of the main taxa in the research area (Taberlet et al. 2007). To construct a local reference database we used taxa commonly identified in the monitoring data of the Aerobiological station in Fondazione Edmund Mach (Appendix 4). The workflow is summarized in Figure D.1.

First, a bioinformatics search was performed in the Genbank database (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov/genbank) for a short fragment of the chloroplast trnL intron (trnL c-h barcode) of species known to occur in the study area, as described in 'La nostra Flora', a reference work for the flora in Trentino (Dalla Fior 1985). In total, 1470 species were searched, belonging to 46 families.

Using the R package 'rentrez' (Winter 2016, https://CRAN.R-project.org/package=rentrez), trnL sequences were downloaded from the database 'Nucleotide' of Genbank using species names as search terms (e.g. '*Abies alba* trnL'). For each of the sequences, the portion between the primers was retrieved and stored in a database including information on the family, genus, species, sequence identifier number in Genbank (gi) and other metadata. The resulting database was then filtered for incorrectly labeled sequences. For this, an exhaustive manual validation was done for synonyms of all related species in Euro+Med PlantBase (http://www.emplantbase.org/home.html) and duplicates were removed. For each family, sequences were then aligned using the 'Muscle' algorithm with the R package 'Bioconductor muscle' (Edgar 2004). Sequences that could not be aligned due to low quality were removed.

After the construction of the local reference database, the taxonomic coverage and the taxonomic resolution of the barcode were investigated. For the latter, a comparison to the trnL P6 loop (g-h) barcode was made, for which a database was also constructed following the steps described above.

Taxonomic coverage: Relevant taxa with low or no availability of trnL sequences were identified and new sequences were generated, especially for taxa of interest in the area (Table D.1). Young leaf samples were collected from different individuals of taxonomically verified plants in San Michele all'Adige (Trentino) and stored at -20ºC until DNA extraction. Total DNA was extracted from 100 mg of leaf tissue, following the manufacturer's protocol of Qiagen kit, and the whole trnL intron was amplified using the primer pair c-A49325 and d-B49863 [5'-CGAAATCGGTAGACGCTACG-3' and 5'-GGGGATAGAGGGACTTGAAC-3'] (Taberlet et al. 2007). The PCR mixture contained 1.25 U Hotmaster Taq polymerase (5 Prime GmbH, Hilden, Germany), 1x of Hotmaster Buffer, 1 mM of dNTPs and 0.5 µM of each primer. The program was set as follows: 94ºC for 2 min, 30 cycles at 94ºC for 30 sec, 55ºC for 15 sec and 65ºC for 2 min, followed by 10 min at 65ºC. Amplicons were Sanger-sequenced in a Genetic Analyzer 3130 (Applied Biosystems, Foster City, CA, USA), and the newly generated sequences were added to already available ones, thus forming the final local reference database. All sequences stored in the database, together with the related information (i.e. gi, family, genus and species), were exported in a fasta file which was used for the final taxonomic assignment. After a bioinformatics search for the trnL c-h barcode, a total of 1188 sequences were retrieved, corresponding to 403 species of 198 genera and 46 families (Figure D.2); 44 additional sequences, corresponding to 26 species, were generated by us (Table D.1).

Taxonomic resolution: We compared the taxonomic resolution from the trnL (c-h) and the trnL P6 loop (g-h), which is a smaller fragment and part of the trnL (c-h). The average length for the c-h barcode was 148 bp, and for the g-h barcode 51 bp. We excluded from the analysis 12 families with a single species and within the rest of the families we investigated the taxonomic resolution of each barcode. We computed with MEGA (Tamura et al. 2013) the mean distance within group (D) and the corresponding standard error (SE), which are presented for both barcodes in Table D.2. Higher genetic distance within family was recorded using the c-h barcode for most of the families (76%). For example, the distance for c-h within Pinaceae is 3.5±1 and within Cupressaceae 2.1±0.8, while for g-h the distance is 1.6±0.6 and 0.8±0.4, respectively.

1. Construct a local reference database

    a. Bioinformatics search of trnL sequences

        Database: 'Nucleotide' (Genbank)

        R package: 'rentrez'
                entrez_search (db='nucleotide', term=search_term)
                entrez_fetch (db='nucleotide', id=search$ids, rettype='fasta')
                search_term= 'species name trnL' (e.g. 'Abies alba trnL')

    b. Raw data processing

        Add metadata to sequences (e.g. family, genus, species).

        Trim sequences so to include only the barcode sequence.

        Remove incomplete and wrongly assigned sequences.

        Within each family, align sequences using R package 'Bioconductor muscle'.

        Remove not aligned (bad quality) sequences.

    c. Addition of new trnL sequences

        Check availability of sequences for all important taxa.

        Generate new trnL sequences by Sanger sequencing when missing.

    d. Construction of the database

        Create a fasta file (*Database.fsa*) with all sequences and some relevant

        information (e.g. >375174864_Pinaceae_Abies_alba).

2. Assign unknown sequences taxonomically

    a. Construction of the BLASTN database

        Format the created database (*Database.fsa*) as needed by BLASTN (*).

    b. Preparation of the unknown sequences (from environmental samples)

        Check the quality of the unknown sequences (Sequencher 5.4).

        Create a fasta file with good quality sequences (*unknown_sequences.fsa*).

    c. Alignment of the unknown sequences to the BLASTN database (**).

    d. Assign taxonomically the unknown sequences

        Export the alignment (*output_file*) in a format that requires relevant

        information about the alignment (e.g. identity percentage, query coverage).

    BLASTN 2.4.0 +
        (*) makeblastdb -in Database.fsa -parse_seqids -dbtype nucl
    (**) blastn -db Database.fsa -query unknown_sequences.fsa -out output_file –outfmt 7

Figure D.1 Workflow followed for the taxonomic identification of pollen from environmental samples, including the construction of a local reference database and the taxonomic assignment of unknown sequences of pollen taxa.

Σχήμα D.1 Βήματα εργασίας που ακολουθήθηκαν για την ταξινομική αναγνώριση της γύρης από περιβαλλοντικά δείγματα. Σε αυτά περιλαμβάνεται η κατασκευή μιας τοπικής βάσης δεδομένων και η ταξινομική ταυτοποίηση άγνωστων αλληλουχιών από τάξα γύρης.
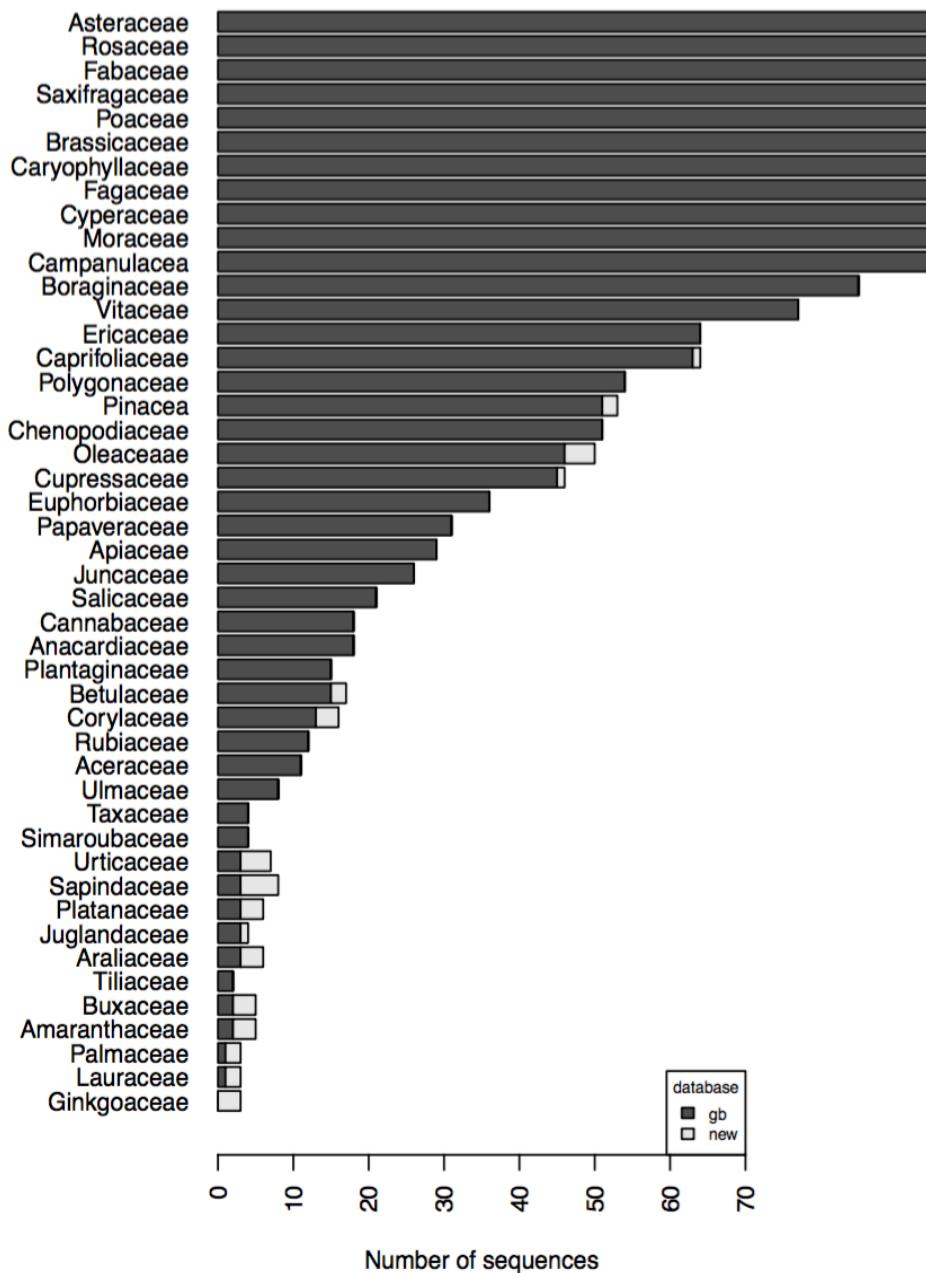
Figure D.2 Number of sequences per family of interest. The sequences that were already available in Genbank are indicated in black and the sequences that were newly produced for the aim of our study are indicated in grey.

Σχήμα D.2 Αριθμός αλληλουχιών ανά οικογένεια. Οι αλληλουχίες που ήταν ήδη διαθέσιμες στην Genbank φαίνονται με μαύρο και οι αλληλουχίες που παράχθησαν για το σκοπό αυτής της μελέτης φαίνονται με γκρι.

Table D.1 List of species for which new trnL sequences were generated with Sanger sequencing and their accession number in Genbank. Where different accession numbers are present for the same species, they refer to different individuals (e.g. *Amaranthus retroflexus*, trnL sequence from 3 individuals). Species are arranged in alphabetical order first according to family.

Πίνακας D.1 Κατάλογος ειδών για τα οποία δημιουργήθηκαν, με αλληλούχιση Sanger, νέες trnL αλληλουχίες, καθω΄ς με και οι αριθμοί πρόσβασης τους στην Genbank. Πολλαπλοί αριθμοί εισαγωγής για το ίδιο είδος αφορούν διαφορετικά άτομα του είδους (π.χ. *Amaranthus retroflexus*, trnL αλληλουχία από 3 άτομα). Τα είδη παρουσιάζονται με αλφαβητική σειρά, πρώτα με βάση την οικογένεια.

Species	Family	Accession number in Genbank
Amaranthus retroflexus	Amaranthaceae	KY313859, KY313860, KY313861
Hedera helix	Araliaceae	KY313862, KY313863, KY313864
Artemisia vulgaris	Asteraceae	KY313902
Ambrosia artemisiifolia	Asteraceae	KY313896
Betula pendula	Betulaceae	KY313865, KY313866
Buxus sempervirens	Buxaceae	KY313867, KY313868, KY313869
Humulus lupulus	Cannabaceae	KY313870
Sambucus nigra	Caprifoliaceae	KY313871
Corylus avellana	Corylaceae	KY313894, KY313895
Ostrya carpinifolia	Corylaceae	KY313872, KY313873
Cupressus arizonica	Cupressaceae	KY313899
Cupressus sempervirens	Cupressaceae	KY313874
Juniperus communis	Cupressaceae	KY313898
Thuja orientalis	Cupressaceae	KY313897
Ginkgo biloba	Ginkgoaceae	KY313875
Juglans regia	Juglandaceae	KY313876
Laurus nobilis	Lauraceae	KY313877, KY313878
Fraxinus ornus	Oleaceae	KY313881, KY313882
Olea europaea	Oleaceae	KY313879, KY313880
Trachycarpus fortunei	Palmaceae	KY313883, KY313884
Cedrus sp.	Pinaceae	KY313887
Larix decidua	Pinaceae	KY313885, KY313886
Platanus sp.	Platanaceae	KY313900
Aesculus hippocastanum	Sapindaceae	KY313888, KY313889
Ulmus sp.	Ulmaceae	KY313901
Parietaria sp.	Urticaceae	KY313890, KY313891, KY313892, KY313893

Table D.2 Distance within family for the ch (D-ch) and the gh (D-gh) barcode, and the standard error (SE-ch) and (SE-gh), given by MEGA. The number of sequences and number of species within family are also presented.

Πίνακας D.2 Απόστασεις μέσα στις οικογένειες για τα barcode ch (D-ch) και gh (D-gh) και τυπικό σφάλμα (SE-ch) και (SE-gh), όπως δόθηκαν από το MEGA. Παρουσιάζεται επίσης ο αριθμός των αλληλουχιών και ο αριθμός των ειδών μέσα στις οικογένειες.

Family	D-ch	SE-ch	D-gh	SE-gh	Sequences	No. Species
Juncaceae	9.9	2.0	9.4	1.6	17	5
Fabaceae	**7.8**	1.4	3.7	1.0	30	18
Caryophyllaceae	**7.7**	1.5	5.2	1.1	29	16
Cyperaceae	**6.7**	1.4	3.6	0.9	23	20
Moraceae	**6.0**	1.0	0.1	0.1	32	2
Polygonaceae	**6.0**	1.5	2.8	0.7	14	6
Taxaceae	**5.5**	1.3	2.1	0.8	10	2
Brassicaceae	4.1	1.2	3.3	1.0	33	6
Rosaceae	3.7	1.1	3.4	1.0	41	21
Ericaceae	**3.7**	1.1	1.4	0.7	28	7
Pinaceae	**3.5**	1.0	**1.6**	0.6	43	17
Chenopodiaceae	3.3	1.4	4.0	1.4	3	2
Urticaceae	3.3	1.1	3.2	1.0	6	2
Euphorbiaceae	3.0	0.8	2.7	0.8	18	16
Caprifoliaceae	2.6	1.0	1.1	0.6	11	8
Cupressaceae	**2.1**	0.8	**0.8**	0.4	14	5
Asteraceae	2.1	0.8	0.2	0.2	60	35
Fagaceae	1.7	0.8	1.2	0.7	9	5
Campanulaceae	1.6	0.7	1.0	0.6	7	5
Apiaceae	1.5	0.7	0.9	0.6	6	5
Boraginaceae	1.3	0.8	0.1	0.1	28	3
Poaceae	1.0	0.6	0.6	0.5	71	10
Rubiaceae	1.0	0.5	0.7	0.4	21	11
Saxifragaceae	0.8	0.2	0.4	0.1	136	6
Salicaceae	0.8	0.5	1.4	0.7	16	8
Aceraceae	0.7	0.7	0.7	0.6	3	3
Betulaceae	0.5	0.5	0.7	0.6	10	5
Oleaceae	0.3	0.2	0.3	0.2	8	5
Tilaceae	0.0	0.0	0.0	0.0	2	2
Corylaceae	0.0	0.0	0.0	0.0	6	3

Chapter E. Developing protocols for molecular analysis

E.1 Introduction

A metabarcoding approach commonly includes as important step to optimize sample processing and DNA extraction (i.e., cell lysis and DNA purification) for the type of sample (Bell et al. 2016).

The aim of this part of the study was to develop protocols for processing aerobiological samples for DNA extraction and metabarcoding analysis and to assess the efficacy of these protocols for the taxonomic assignment of airborne pollen. For this aim, we designed a series of experiments that examine variables that optimize DNA yield during DNA extraction (e.g. extraction kit, disruption method) of pollen. Using the classical morphological pollen analysis as a benchmark and having the local reference database of the study, we examined the conditions under which DNA metabarcoding (combined here with Sanger-sequencing) is applicable across a complexity of samples: from those containing only pure single-species pollen to complex aerobiological samples, collected by both gravimetric (Tauber) and volumetric (Hirst-type) air samplers (henceforth environmental samples).

E.2 Methodology

E.2.1 Study area

This study was conducted in Trentino (45°40′-46°30′N, 10°30′-12°00′E), a mountainous region of northern Italy, with a total surface area of 2,607 km^2 and an elevation ranging from 65 m to 3,764 m above sea level. The region is characterized by diverse phytoclimatic types.

The Aerobiological Monitoring Centre of Fondazione Edmund Mach is situated in the valley of the river Adige, in San Michele all'Adige, 220 m above sea level. The aerobiological sampling site is in proximity to vineyards and apple orchards, which cover 44% of the surrounding area. A further 8% is covered by herbaceous weeds from the genera *Parietaria* (*P. diffusa*, *P. officinalis*), *Artemisia* (*A. absinthium*, *A. vulgaris*, *A. verlotorum)* and *Ambrosia* (*Ambrosia artemisiifolia*), while at higher elevations, 48% of the territory is forested: 24% mixed (e.g. *Pinus sylvestris*, *Ostrya carpinifolia*, *Fraxinus ornus*, *Quercus pubescens*), 11% coniferous (mainly *P. sylvestris*, *Pinus nigra* and *Picea excelsa*) and 8% broad-leaved (e.g. *Fagus sylvatica*, *F. ornus*, *O. carpinifolia*, *Q. pubescens*); the remaining 5% consists of semi-natural areas, transitional woodland, shrubs, and sparsely vegetated areas (Cristofori et al. 2010).

E.2.2 Collection of samples, sample processing and microscopic analysis

Environmental samples were collected at the Aerobiological Monitoring Centre of Fondazione Edmund Mach. These samples were collected by means of volumetric and gravimetric samplers. To examine how close are the results obtained with volumetric and gravimetric samplers, we made a number of experiments, in which we used the two types of samplers concurrently.

Two sets of data had to be collected for each type of sampler, one for microscopic and one for molecular analysis. Regarding the volumetric samplers, we had available one Lanzoni sampler (VPPS 2000, Lanzoni, Bologna, Italy) and one Burkard sampler (Sporewatch Electronic Spore & Pollen Sampler, Burkard Manufacturing Rickmansworth, Hertsfordshire, England), both of Hirst-type (Hirst 1952). To make use of their data, we had first to make sure that the results obtained with either of them were the same. Daily pollen data obtained over a 5-month period (10 June to 3 November, 2013) were compared and showed a good correlation (Spearman's Rho = 0.91; p<0.001), with no significant differences in terms of pollen load (Mann-Whitney U test). Therefore, we could use these two Hirst-type traps and accept pollen samples from them as replicate samples.

The two Hirst-type collectors were set 10 m off the ground, next to each other (Figure E.1a). The pollen that was to be analyzed both with the classical microscopical approach and with molecular methods was collected within the period 23 February to 2 March. Pollen grains were collected on a silicone-coated adhesive tape (Melinex; DuPont Teijin Films Luxembourg, SA, Luxembourg City, Luxembourg). The Burkard tape was cut in two longitudinal halves, which were analyzed as replicates by molecular techniques. The Lanzoni tape was analyzed by light microscopy. The gravimetric samplers, Tauber traps (Tauber 1974), were set two meters away from the volumetric samplers. Their aperture, 0.5 m from the ground (Figure E.1b), was equipped with a collar, from which air flows into the trap, and covered by a 5 mm mesh to prevent insects and/or other large particles entering the trap. Pollen was collected in 700 ml of preservative solution (1:1:1 water, alcohol, glycerol, plus 2 g l^{-1} phenol). Three gravimetric traps were set up concurrently; two were analyzed as replicates by molecular techniques and the third by microscopic techniques. The samples collected from them correspond to a two-month period (7 August to 14 October, 2015).

Figure E.1 (a) Volumetric (Burkard and Lanzoni) and **(b)** gravimetric samplers (Tauber)

Σχήμα **E.1** Παγίδες δειγματοληψία **(a)** ογκομετρικές (Burkard και Lanzoni) και **(b)** καθίζησης (Tauber)

(a)   (b)

Pollen pellets for molecular and microscopic analysis were retrieved using the following protocol.

Filtration (for Tauber trap samples only): Samples were pre-filtered through a 200 µm metal mesh sieve (Retsch, Haan, Germany) to remove large particles (e.g. small insects, plant remains) and pollen was collected on a 5 µm mixed cellulose ester filter (47 mm diameter, Merck Millipore Ltd, Cork, Ireland), using a vacuum pump filtration system (Sigma-Aldrich, Milan, Italy). When samples were prepared for microscopic analysis, a counting marker (*Lycopodium* spore tablets, Batch 3862, Lund University, Lund, Sweden) was added before filtration. Filters were dried for 2 to 3 h at 65°C, and either immediately processed or stored at -20°C until further analysis.

Pellet preparation (for Tauber trap filters and longitudinal halves of Burkard tape corresponding to one week): Samples were placed into 15 ml glass tubes (with the tape samples first cut into smaller pieces) and 5 ml of acetone were added. The samples were vortexed for 2-3 min to dissolve the filters and elute the tapes and centrifuged for 3 min at 2,300 rpm. The same vortexing and centrifuging conditions were applied for the subsequent steps. After centrifuging, the supernatant was discarded and 1 ml of acetic acid was added; vortexing and centrifuging steps followed. After discarding the supernatant, two or four washing steps were performed to clean the pellet using 2 ml of distilled water, plus one drop of ethanol to reduce the surface tension; vortexing and centrifuging steps followed. The supernatant was discarded and the pellet was re-suspended in 1 ml of distilled water, transferred into 2 ml tubes, and centrifuged for 3 min at 13,500 rpm. For the two Tauber filters and the two longitudinal halves of the Burkard tape, the supernatant was discarded and the pellets were stored at -20°C until molecular analysis. For the pellet obtained from the third Tauber filter, 0.5 ml of the supernatant were kept, two drops of glycerol were added and the samples were stored at 4°C until analysis by light microscopy.

A classical morphological pollen analysis was performed using an optical microscope (Leitz Diaplan, Ernst Leitz Wetzlar, Wetzlar, Germany) at 400x magnification

for both sample types. For the Tauber sample, at the end of the pelleting procedure, a small aliquot of the pellet was transferred onto a microscope slide and colored with basic fuchsine. A minimum of 400 pollen grains were counted and identified at the microscope and the total pollen was then calculated by taking into account the number of counted markers (Faegri & Iversen 1989). For the volumetric sampler (Lanzoni), daily segments of the tape were mounted on slides, colored with basic fuchsine, and four horizontal continuous sweeps were analyzed (14% of the total area), following the European standard (UNI CEN/TS 16868:2015), and the weekly sum was calculated. This method allowed a quantitative estimation of the pollen present in the samples (Table E.1).

E.2.3 DNA extraction experiments

Experiments were designed to define the DNA extraction conditions yielding the highest DNA quantity from pollen samples (Figure E.2). Three replicates were performed for DNA extraction from single-species pollen samples, and two for environmental samples. DNA yield was measured by a Qubit 2.0 Fluorometer (Life Technologies, Thermo Fisher Scientific Inc.). The final elution volume was 100 μl. Two different kits were tested, DNeasy Plant mini kit (Qiagen, GmbH, Hilden, Germany) (henceforth Qiagen kit), which is based on silica gel spin column technology, and Nucleomag kit (Macherey-Nagel, Düren, Germany), which is based on magnetic bead technology, using the automated DNA extraction systems Qiacube (Qiagen) and Kingfisher (Thermo Fisher Scientific, Waltham, MA, USA), respectively. Samples for DNA extraction were of increasing complexity (see Table E.1): (a) pure single-species pollen, (b) single-species pollen added on sampling tapes or in Tauber solutions, (c) environmental samples.

Tests on pure single-species pollen: The best conditions for pollen disruption and DNA extraction kit were evaluated. The samples consisted each of 2.5 mg of *Corylus avellana* L., *Juniperus communis* L. or *Artemisia vulgaris* L. pure pollen that was collected directly from flowers and stored at 4ºC before analysis. This quantity corresponds to approximately 260,000-460,000 pollen grains, depending on the species, as estimated by a Fuchs-

Rosenthal counting chamber (HBG Henneberg-Sander GmbH, Giesen, Germany). The quantity was chosen to reflect the effective quantity of pollen grains found in seasonal Tauber traps located within various NATURA 2000 habitat sites, as estimated in a preliminary study (15,000-360,000 pollen grains per Tauber trap, in spring, higher to lower altitudes) using *Lycopodium* as a counting marker.

Prior to DNA extraction, to improve cell lysis by mechanical disruption of cell walls, high-energy agitation with beads was applied with a Retsch MM200 mixer mill. The beating power of glass beads was compared to that of steel beads. In an additional test, we froze samples in liquid nitrogen for 30 s prior to bead beating, as suggested by the Qiagen handbook. For each sample, beads were added as follows: 1 steel bead with a diameter of 5 mm (Qiagen) or, alternatively, a mixture of 0.5 g of glass beads (0.3 g of 212-300 μm in diameter and 0.2 g of 425-600 μm in diameter) (Sigma-Aldrich). All samples were ground for 1 min at 30 Hz, in two steps. All possible combinations of the disruption method and the extraction kit variables were tested.

Microscope analysis of pollen suspensions was carried out before and after lysis under the conditions found optimal to validate the lysis efficiency of the method across all three taxa. For this, we prepared the pollen suspensions as suggested by Kraaijeveld et al. (2015), and then used a Fuchs-Rosenthal chamber to count ten microscope fields before and after lysis for each pollen sample.

DNA extraction under 'optimal' conditions: The optimal conditions of each step were applied on more complex samples: (a) Single-species pollen on tape or in Tauber solution, (b) two-species mixture of pure pollen, (c) diluted single-species pollen, (d) environmental samples.

(a) Single-species on tape or in Tauber solution

Here we simulated the conditions applied in the field for volumetric and gravimetric collection of airborne pollen and we tested different extraction conditions to establish the optimal ones on this sample type. 2.5 mg of *J. communis* pure pollen, were distributed (i) on a section of aerobiological tape corresponding to one day (48 mm) or (ii) in a Tauber trap aqueous solution. For the preparation of the tape and the Tauber solution samples

and for their processing, we followed the protocol described above for environmental samples (section E.1.1), and DNA was extracted from the obtained pellet.

We also examined if the DNA yield is negatively influenced by chemicals used in the sample processing or by filter remains (in the case of Tauber samples). For this purpose, we applied two or four washing steps during sample processing, before DNA extraction with any of the kits and under the optimal disruption conditions.

(b) Two-species mixture of pure pollen

Here we tested the optimal protocol on a mixture of *C. avellana* and *J. communis* pure pollen (50:50). For this, we added 2.5 mg of the two pollen species (of the same size group) in a proportion of 50:50.

(c) Single-species pollen of diluted concentrations

We used a range of starting concentrations of *C. avellana* pollen to verify its efficacy on low quantities of pollen. For this experiment, we added 25 mg of *C. avellana* in 10 mL of water, we estimated the concentration with a Fuchs Rosenthal chamber, and then diluted at three different concentrations (1:10, 1:100 and 1:1000), corresponding respectively to 26,000, 2,600 and 256 total pollen grains, for subsequent DNA extraction.

(d) Environmental samples

We examined the efficiency of the optimal conditions identified for single-species pollen when applied to the complex pollen spectrum of environmental samples. These are characterized by several pollen species and other bioparticles and possibly suffer from DNA degradation processes, due to their prolonged exposure in the field (one week for the tape samples and two months for the Tauber ones). For this experiment, two Tauber traps and one Burkard tape corresponding to one week were collected and processed as described above (section E.1.1), and DNA was extracted from the obtained pellet.

E.2.4 PCR, cloning and sequencing

Pollen DNA was amplified with c-A49325 and h-B49466 trnL primers [5'-CGAAATCGGTAGACGCTACG-3' and 5'-CCATTGAGTCTCTGCACCTATC-3'] (Taberlet

et al. 2007) produced by Sigma-Aldrich. DNA amplification was carried out in a final volume of 50 µl using 2 µl of DNA as a template. The amplification mixture contained 1.25 U GoTaq Polymerase (Promega, Madison, WI, USA), 1x of GoTaq Flexi Buffer, 3 mM of MgCl2, 0.2 mM of dNTPs and 0.2 µM of each primer. All PCR amplifications were carried out on a Veriti 96 well thermal cycler (Applied Biosystems), with the following program: 2 min at 95ºC and 40 cycles of 15 sec at 95ºC, 15 sec at 52ºC and 30 sec at 72ºC, followed by 5 min at 72ºC. Band sizes and concentrations were checked on a gel electrophoresis using QIAxcel (Qiagen), and analyzed by QIAxcel ScreenGel Software.

For samples containing pollen from multiple species (mix of two species and environmental samples), cloning prior to sequencing was performed. First, PCR products were run on a 2% agarose gel and bands of the adequate size were excised and purified with the commercial QIAquick gel extraction kit (Qiagen). Cloning was then performed, using the TOPOTA Cloning Kit, Dual Promoter, with pCRII-TOPO Vector and One Shot Mach1 T1 Phage-Resistant Chemically Competent E. coli (Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocol.

For all positive colonies, DNA amplification was carried out with the M13 forward and reverse primers [5'-GTAAAACGACGGCCAG-3' and 5'-CAGGAAACAGCTATGAC-3'] (Sigma-Aldrich) in a final volume of 20 µl, using 1 µl of DNA as template. The PCR mixture contained 1.25 U Hotmaster Taq polymerase (5 Prime GmbH, Hilden, Germany), 1x of Hotmaster Buffer, 1 mM of dNTPs and 0.5 µM of each primer. The program was set as follows: 94ºC for 2 min, 35 cycles at 94ºC for 30 sec, 55ºC for 15 sec and 65ºC for 2 min, followed by 10 min at 65ºC. The results of this amplification were verified on Qiaxcel software for presence/absence of amplification, size and concentration of bands. For sequencing, we selected about 30 amplicons of different sizes to ensure a good coverage of the species present in the samples.

For all samples, we purified the PCR products using ExoSap-IT (Amersham Pharmacia Biotech, Uppsala, Sweden). The purified DNA was Sanger-sequenced using 3.2 µM of the M13 reverse primer for the cloned samples and 3.2 µM of trnL primers for the rest of the samples and a BrightDye Terminator 2.5X Premix Cycle sequencing mix

(Nimagen, Nijmegen, Netherlands). All samples were loaded in a Genetic Analyzer 3130 (Applied Biosystems).

E.2.5 Taxonomic assignment

The taxonomic assignment was done with BLAST 2.4.0+ software (Camacho et al. 2009) by comparison of the new unknown sequences to our local reference database (Chapter D, Figure D.1), which was used as a BLASTN database. After obtaining the unknown sequences, we used Sequencher version 5.4 sequence analysis software (Gene Codes Corporation, Ann Arbor, MI, USA) in order to assess the quality of the sequences and further process them. All good quality sequences (i.e. those with unambiguous base peaks in the electropherogram of Sequencher) were stored in a fasta file. Hence, each of the unknown sequences was aligned to the sequences in the local reference database and information about the alignment was requested (e.g. identity percentage and query coverage) to taxonomically assign the sequences. Sequences with more than 98% identity score and query coverage were assigned to the presumed taxa.

E.2.6 Statistic analysis

The DNA yield data were analyzed using R software (version 3.3.2; R core team 2016). A three-way ANOVA for all effects and interactions was applied on the DNA extraction data from pure single-species pollen, with independent variables being the kit, the freezing step and the bead material, while the dependent variable was the DNA yield. To compare results from pure single-species pollen samples and single-species tape and Tauber samples, we applied a one-way ANOVA, using the type of sample as the independent variable and the DNA yield as the dependent variable. A three-way ANOVA for factor interactions was also applied on data from single-species tape or Tauber samples with the independent variables being the kit, the type of sample and the number of washing steps, and the dependent variable the DNA yield.
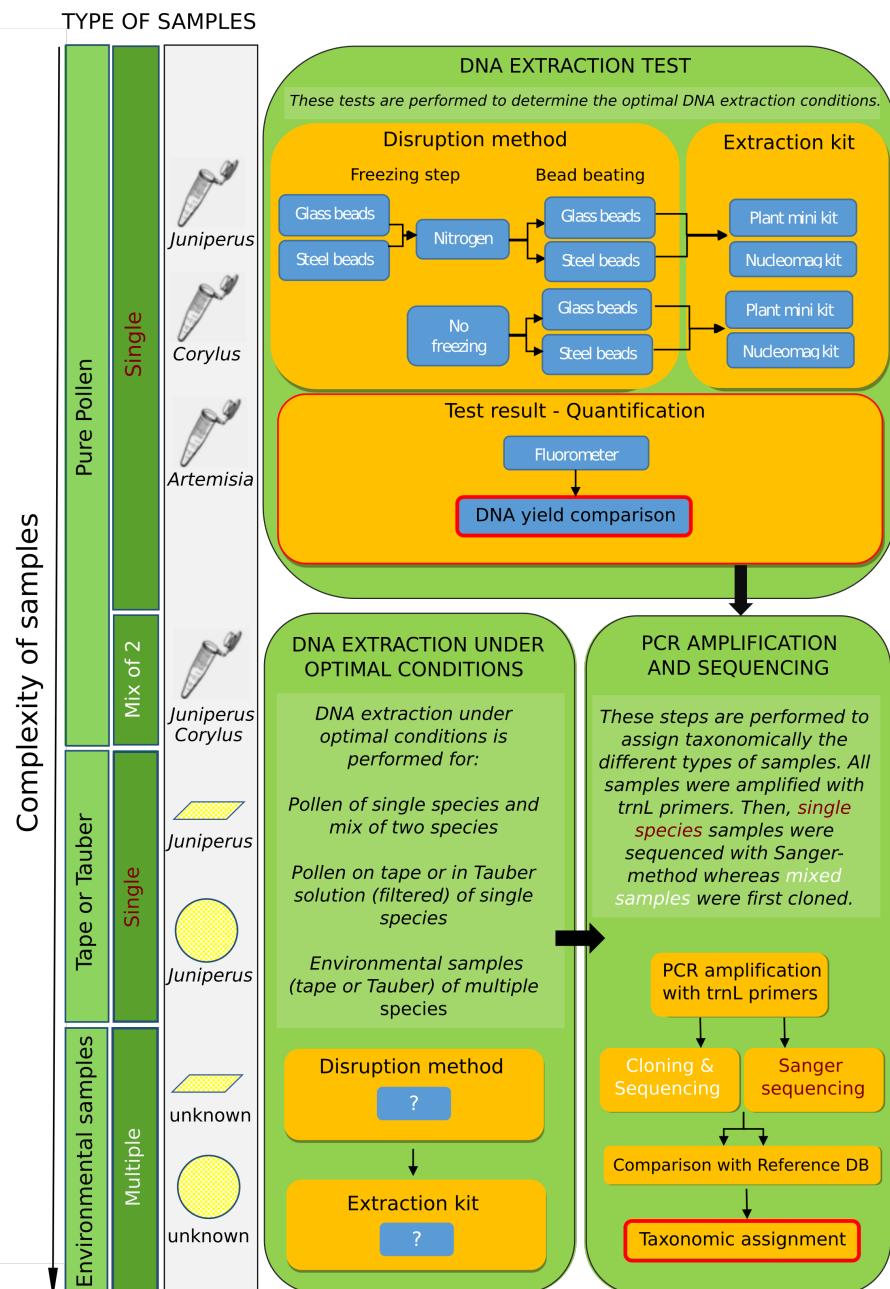
Figure E.2 Workflow of the experiments performed to determine the optimal DNA extraction conditions using pure pollen in single species and application of the optimal conditions in more complex samples (two-species mix of pollen, single species pollen fixed on tape and in Tauber solution and complex environmental samples collected both on tape and in Tauber medium). All samples are finally amplified using plant-specific primers for taxonomic assignment.

Σχήμα E.2 Βήματα εργασίας που ακολουθήθηκαν για τα πειράματα ανεύρεσης των βέλτιστων συνθηκών απομόνωσης DNA χρησιμοποιώντας γύρη ενός είδους και περαιτέρω εφαρμογή των βέλτιστων συνθηκών σε πιο περίπλοκα δείγματα (γύρη από δύο είδη σε μίξη, γύρη μοναδικού είδους εφαρμοσμένη σε ταινία και διάλυμα Tauber και περιβαλλοντικά δείγματα που συλλέχθηκαν σε ταινία και διάλυμα Tauber). Όλα τα δείγματα ενισχύθηκαν με εκκινητές για φυτά για την τελική τους ταξινομική αναγνώριση.

E.3 Results

E.3.1 Selection of 'optimal' DNA extraction protocols

The 'optimal' conditions were selected by averaging results for the three species (*Corylus avellana, Juniperus communis* and *Artemisia vulgaris*), as our aim was to work with complex mixtures of multiple species (environmental samples). Results of the statistical tests and of the obtained DNA yields are shown in Tables E.2 and E.3, respectively. The DNA yields were significantly different between extraction kits (ANOVA; $p<0.05$) and bead materials (ANOVA; $p<0.001$). In particular, the highest DNA yield was obtained with a steel bead disruption step and the 'Nucleomag' extraction kit. Adding a freezing step did not influence the yield. Finally, the ANOVA showed no significant interactions between the variables examined.

Pollen lysis took place effectively for all three species. Before lysis, the concentration was found to be 408 pollen grains μl^{-1} for *C. avellana*, 480 pollen grains μl^{-1} for *J. communis* and 576 pollen grains μl^{-1} for *A. vulgaris*. After lysis the concentrations were 24 pollen grains μl^{-1} (95% lysis), 0 pollen grains μl^{-1} (100% lysis), 56 pollen grains μl^{-1} (90% lysis), respectively, resulting in a >90% lysis for all taxa.

When mixing different taxa (*J. communis* and *C. avellana),* we had a good recovery of DNA. Finally, we found that different dilutions of pure *C. avellana* pollen (starting quantity: 256,000 pollen grains) did not affect the DNA yield significantly, even when pollen was very diluted. DNA yields are reported in Table E.2.

When applying the same extraction protocol to pollen of *J. communis* from tape (as in volumetric sampling) or Tauber solution (as in gravimetric sampling), the DNA yield decreased significantly compared to pure pollen samples in both cases (ANOVA; $p<0.05$) (Figure E.3a).

For both sampling methods (tape and Tauber trap), there was a significant increase in DNA yield when the Nucleomag kit was used and, in case of Tauber trap samples, DNA yield increased when the washing steps were doubled (ANOVA; $p<0.001$);

no significant difference of this type was found for the tape samples (Figure E.3b).

The 'optimal' conditions were defined as those including disruption of the pollen cell wall with steel beads, and DNA extraction with the Nucleomag kit for both Tauber and tape samples. Four washing steps during the sample processing are suggested for Tauber samples, while for tape samples two washing steps are enough.

The applied 'optimal' conditions resulted into lower DNA yield for environmental samples than that obtained from single-species pollen on tape or in Tauber solution (Table E.2).

E.3.2 Taxonomic identification

PCR results observed in the electropherogram of Qiaxcel always showed well-defined fragments, without primer dimers, indicating good quality of extracted and amplified DNA. No PCR inhibition was detected. For the environmental samples, 124 positive colonies were recovered from the tape and 117 colonies from the Tauber solution; 31 and 28 amplicons, respectively, were selected for sequencing. The amplified fragments were successfully sequenced by Sanger-sequencing for 99% of all samples (n=155), with only one bad quality sequence in terms of ambiguities in the base peaks, which was excluded from the analysis.

For pure single-species pollen, the extraction test resulted in a correct taxonomic assignment for 100% of the samples (n=72, 3 species, 3 replicates, 8 tests). *J. communis* tape or Tauber samples were correctly assigned taxonomically for 100% of the samples (n=24, 1 species, 2 sample types, 3 replicates, 4 tests). For the mix *C. avellana* and *J. communis* we sequenced 6 cloned amplicons obtaining 5 sequences of *C. avellana* and 1 of *J. communis*.

The taxonomic assignment of unknown sequences from environmental samples resulted in 7 different taxa for tape and 3 different taxa for Tauber samples. All sequences were identified with a 100% identity match on the whole sequence length (100% query coverage), except for *Urtica dioica* whose taxonomic identity was 99.2%, with a coverage of 99%. When compared to light microscope results (Table E.4), 75% and 37% of the taxa

identified by microscope were revealed by metabarcoding for the volumetric and the gravimetric sampling, respectively. *Cedrus* was dominant in the sequencing results of the Tauber environmental sample.
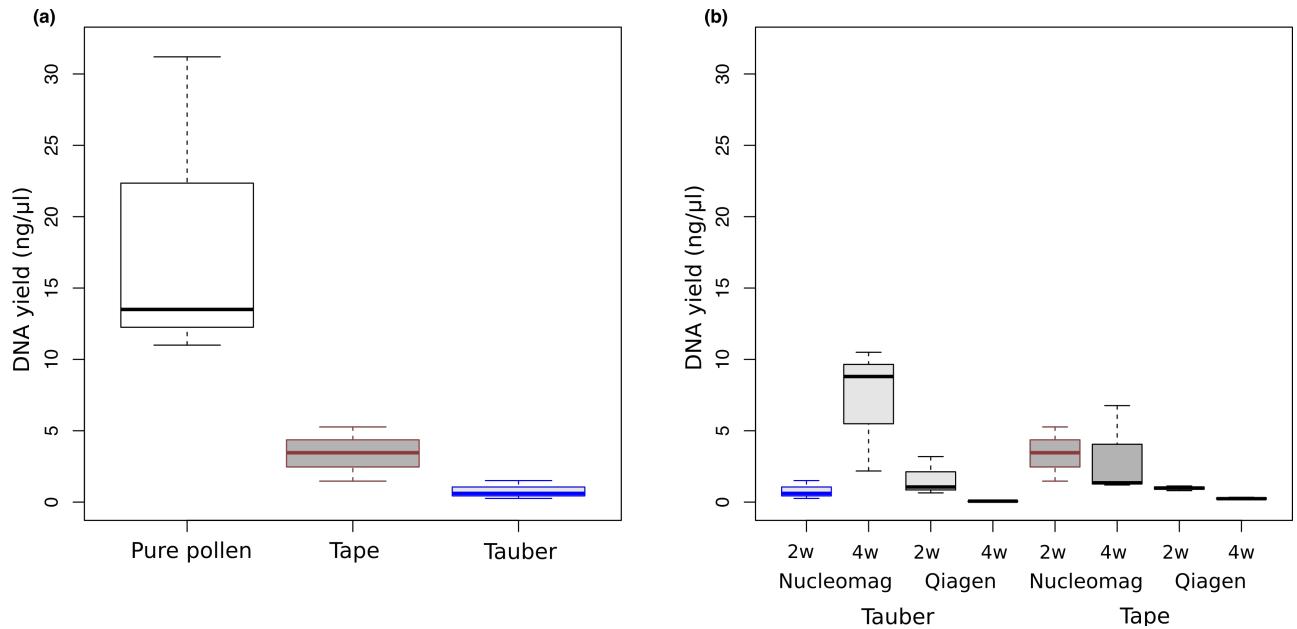
Figure E.3 DNA yield for the different types of *Juniperus communis* samples. The boxplots give the 25th and 75th percentile (bottom and top of the box, respectively) and the median (black, brown and blue thick line). The upper and lower whiskers correspond to the maximum and minimum obtained DNA yield values, respectively. Boxplot **(a)** provides DNA yields of the extraction for pure single-species pollen (white box), single-species pollen from tape (grey box) and Tauber solution (light grey box) after two washing steps, Nucleomag kit and steel beads. Boxplot **(b)** provides DNA yield of single-species pollen from tape (grey box) and Tauber (light grey box) samples after two (2w) and four (4w) washing steps for two extraction kits (Nucleomag and Qiagen) and steel beads.

Σχήμα E.3 Απόδοση DNA για τους διαφόρους τύπους δειγμάτων *Juniperus communis*. Τα boxplots δίνουν το 25ο και 75ο εκατοστημόριο (άνω και κάτω περιοχή, αντίστοιχα) και τη διάμεσο (μαύρη, καφέ και μπλε παχιά γραμμή). Τα ανώτερα και κατώτερα άκρα αντιστοιχούν στη μέγιστη και ελάχιστη απόδοση, αντίστοιχα. Το boxplot **(a)** δίνει την απόδοση DNA για γύρη *από ένα μόνο είδος (λευκό) και από ένα είδος σε ταινία (γκρι) ή σε διάλυμα Tauber (ανοιχτό γκρί) μετά από δύο πλυσίματα και χρήση Nucleomag kit και χαντρών χάλυβα.* Το boxplot **(b)** δίνει την απόδοση DNA για γύρη *από ένα μόνο είδος από δείγματα ταινίας (γκρι) και Tauber (ανοιχτό γκρι) μετά από δύο (2w) και τέσσερα (4w) πλυσίματα και χρήση των δύο kits (Nucleomag και Qiagen) και χαντρών χάλυβα.*

Table E.1 Features of pollen samples used for microscopic and molecular analyzes.

Πίνακας E.1 Χαρακτηριστικά των δειγμάτων γύρης που χρησιμοποιήθηκαν για μικροσκοπικές και μοριακές αναλύσεις.

Sample type	Sample description	Contributing taxa	Estimated no. of pollen grains	Sampling period	Complexity factors
Pure, single-species pollen	Collected directly from flowers and used for molecular analysis	*Corylus avellana* *Juniperus communis* *Artemisia vulgaris*	260,000 400,000 460,000		none
Single-species pollen on Tape and in Tauber	Simulations of volumetric and gravimetric sampling methods used for molecular analysis	*Juniperus communis*	400,000		Sampling medium
Environmental sample on tape	Volumetric samples collected by Burkard trap and used for molecular analysis and by Lanzoni trap used for microscopic analysis	Pollen from several species	11,500	23/02/2015 to 02/03/2015	Sampling medium, degradation processes and occurrence of other bioparticles
Environmental sample in Tauber solution	Gravimetric samples collected by Tauber traps; two traps for molecular analysis and one for microscopic analysis	Pollen from several species	79,000	07/08/2015 to 14/10/2015	Sampling medium, degradation processes and occurrence of other bioparticles

Table E.2 Variables examined to identify the 'optimal' extraction conditions. The selection criterion was a significant increase in DNA yield (p<0.05); ns = no significant effect.

Πίνακας Ε.2 Μεταβλήτες που εξετάστηκαν για τον προσδιορισμό των 'βέλτιστων' συνθηκών απομόνωσης DNA. Το κριτήριο επιλογής ήταν η σημαντική αύξηση στην απόδοση του DNA (p<0.05); ns = μη σημαντική επίδραση

Protocols	Tested Variables	Selected option	Sample type
DNA extraction	Freezing step: with or without Bead material: glass or steel Extraction kit: Qiagen or Nucleomag	ns Steel beads (p<0.001) Nucleomag kit (p<0.05)	Pure pollen of *Juniperus communis, Corylus avellana, Artemisia vulgaris*
Sample processing	Washing steps: 2 or 4	4 washing steps in Tauber solution (p<0.001); ns for tape	Pure pollen of *J. communis* on tape and in Tauber solution

Table E.3 DNA yields with the 'optimal' extraction conditions (i.e. steel beads, Nucleomag kit, four washing steps for Tauber solution, two washing steps for tape) for each type of sample: pure single-species pollen of three different taxa, pollen of *J. communis* on tape and in Tauber solution, environmental samples.

Πίνακας Ε.3 Απόδοση DNA με 'βέλτιστες' συνθήκες απομόνωσης (δηλαδή χάντρες χάλυβα, Nucleomag kit, τέσσερα βήματα πλυσίματος για Tauber διάλυμα, δύο βήματα πλυσίματος για την ταινία) για κάθε τύπο δείγματος, συγκεκριμένα γύρης από ένα μόνο κάθε φορά από τα τρία είδη που εξετάστηκαν, γύρης του *J. communis* σε ταινία και διάλυμα Tauber, καθώς και γύρης από περιβαλλοντικά δείγματα.

Species	Sample type	Average DNA yield
Artemisia vulgaris	Pure pollen	2.66 ± 1.84
Corylus avellana	Pure pollen	0.31 ± 0.14
Juniperus communis	Pure pollen	18.56 ± 11.01
J. communis, C. avellana	Mixed pure pollen	13.06 ± 4.65
Corylus avellana	Pure pollen (dilution 1:10)	0.24 ± 0.2
Corylus avellana	Pure pollen (dilution 1:100)	0.14 ± 0.01
Corylus avellana	Pure pollen (dilution 1:1000)	0.14 ± 0.01
Juniperus communis	Pure pollen in Tauber solution	8.8 ± 4.39
Juniperus communis	Pure pollen on tape	3.1 ± 3.16
various	Environmental sample in Tauber solution	1.36 ± 0.21
various	Environmental sample on tape	0.2 ± 0.02

Table E.4 Pollen taxa identified with the molecular and microscopic methods from samples subject to same environmental and processing conditions.

Πίνακας E.4 Ταχα γύρης που ταυτοποιήθηκαν με μοριακές και μικροσκοπικές μεθόδους από δείγματα που συλλέχθηκαν κάτω από ίδιες περιβαλλοντικές συνθήκες και επεξεργάστηκαν με ίδιο τρόπο.

Environmental	Molecular analysis	Microscopic analysis
On tape	*Juniperus communis*	Cupressaceae-Taxaceae
	Thuja orientalis	Cupressaceae-Taxaceae
	Ulmus	*Ulmus*
	Alnus	*Alnus*
	Corylaceae	*Corylus*
	Poaceae: *Lolium, Festuca*	Poaceae
	Asteraceae: *Artemisia., Achillea*	
	Leucanthemum vulgare	---
	Tanacetum vulgare	
	---	*Ostrya*
	---	Urticaceae
	---	*Fraxinus excelsior*
In Tauber solution	*Urtica dioica*	Urticaceae
	Juniperus communis	Cupressaceae-Taxaceae
	Cedrus	Pinaceae
	---	Chenopodiaceae-Amaranthaceae
	---	Poaceae
	---	*Artemisia*
	---	*Plantago*
	---	Cannabaceae

E.4 Discussion

In this study, we optimized a DNA extraction protocol that maximizes the DNA yield from pollen collected by gravimetric and volumetric samplers. With the exception of the cell lysis, the steps of this procedure can be easily automated (automatic DNA extraction instrument), making the procedure fast and cost-effective. The method worked properly

for all tested pollen types, although the DNA yield was different across taxa in single-species samples. This is probably related to the species-specific starting quantity of DNA. It is reported, for example, that conifers have a large, highly repetitive genome (De La Torre et al. 2014) and they are especially rich in cpDNA because of its exceptional paternal inheritance (Parducci et al. 2005). This could explain the higher DNA yield of the coniferous *J. communis* (compared to *A. vulgaris* and *C. avellana*). Cell wall structure also influences the DNA extraction (Kraaijeveld et al. 2015), as seen by the 100% lysis efficiency of *J. communis* pollen grains, characterized by a very thin exine layer, which leads to higher DNA yields than in case of *C. avellana* and *A. vulgaris* pollen grains, characterized by higher resistance of the cell wall.

We found that the total amount of DNA recovered in the Tauber solution became significantly higher with extra washing steps, while it doesn't seem to add any advantage on tape samples (test performed on *J. communis* pollen). This suggests that residues related to the medium of the gravimetric trap negatively affected the DNA extraction, probably due to their interactions with kit chemicals.

The DNA extraction conditions that were considered optimal after the exploratory experiments, when applied on environmental samples, resulted in lower DNA yield. This may be due to at least three reasons: (i) the concentration of pollen grains in the environmental samples was lower than in single-species samples, (ii) environmental DNA degradation may have happened and (iii) other biological particles in the sample (e.g. fungi, bacteria) may have negatively affected the DNA extraction steps. For the sample preparation, we tried to minimize material loss selecting a filter that dissolves in acetone, which we used also to elute the tape. Finally, by applying the same conditions in highly diluted samples we verified that the starting quantity is not a limiting factor for the efficiency of the protocol.

Sanger-sequencing on DNA extracted from single-species samples simulating field samples collected volumetrically and gravimetrically resulted in a correct taxonomic assignment on all samples of this type. For environmental samples, we adopted a semi-metabarcoding approach by using (i) universal primers for plants, which allowed to

amplify multiple species at the same time without the need of taxon-specific amplification and (ii) the traditional molecular-based approach of cloning and sequencing of several amplicons, partially overcoming the limit of Sanger-sequencing, which can only sequence single-species, individually. We used for comparison the results of classical morphological identification of pollen grains from replicate environmental samples. In the case of the aerobiological tape samples, the trnL c-h barcode identified the majority of the taxa that were determined by morphological analysis, whereas for Tauber solution samples it was less efficient.

For both Tauber and tape samples, our molecular method provided on average a higher taxonomic resolution than microscopy. For example, pollen identified under the microscope as Cupressaceae-Taxaceae type was assigned to *J. communis* and *Thuja orientalis* by molecular analysis. For big families, such as Poaceae and Asteraceae, the molecular method gave two and five assigned taxa, respectively. For instance, after morphological analysis the Poaceae pollen was assigned to the genera *Lolium* and *Festuca*, by molecular analysis. Our results, even though based on Sanger-sequencing, confirmed a resolution at the genus level for grasses, as showed by Kraaijeveld et al. (2015). In contrast, within the Corylaceae family, the trnL could not discriminate among *Ostrya carpinifolia*, *Carpinus betulus* and *C. avellana*, whereas microscopy could assign pollen to the species level. For all taxa, the molecular method was able to assign sequences at least to the family level; this was not the case for the light microscope method which could not distinguish Chenopodiaceae from Amaranthaceae or Cupressaceae from Taxaceae.

The most effective approach to take advantage of the efficiency of DNA metabarcoding is by determining a large number of different sequences, by means of NGS. However, the successful outcome of any NGS approach is highly dependent on DNA quality and quantity, i.e. sufficient amounts of high-quality nucleic acids, which will be representative of the pollen cells in the sample, for subsequent library preparation (Bell et al. 2016). Therefore, we developed protocols for obtaining high quality pollen DNA from environmental samples, without PCR inhibitors or sequence ambiguities after Sanger-sequencing. Although we used a suboptimal methodology for taxonomic assignment, i.e.

Sanger-sequencing of cloned PCR products from each environmental sample, nonetheless, the final outcome is encouraging: good DNA quality can be efficiently retrieved from a complex mixture of pollen grains and other bioparticles. Moreover, as expected, the molecular analysis allowed higher taxonomic resolution compared to analysis by light microscope.

The most critical issue is the low efficiency in the identification of pollen from gravimetric samples: only 37% of the morphologically identified taxa were detected. The volumetric sampling has advantages over the gravimetric one regarding the DNA quality as the tape is exposed in the field only for a short period, one week, whereas the Tauber traps normally remain for longer, two months in our experiments. Moreover, the aqueous medium of the Tauber trap can lead to increased DNA degradation compared to the tape of the volumetric sampler, which is an inert medium where particles attach. The medium of the gravimetric sampler is expected to be more prone to oxidative reactions and microorganism proliferation resulting in higher rates of DNA degradation and accumulation of molecules different than those of pollen. Secondary metabolites, produced by degradation of biological particles, may interact negatively with chemicals used in DNA extraction and amplification. Also, since the Tauber traps are closer to the ground level it cannot be excluded that more particulate matter is collected in the medium. This is another factor potentially affecting DNA amplification. The non-proportional representation of taxa in the results from molecular analysis of Tauber samples could be attributed, at least to a certain degree, to the high presence of conifers in the area, *Cedrus* in particular, that are reported to have high amounts of cpDNA (Parducci et al. 2005), thus affecting the sequencing results.

Several lines of evidence suggest that a NGS-based metabarcoding approach could significantly increase the information from environmental samples in terms of number of identified taxa (Bell et al. 2016). Also, for a better taxonomic assignment in families, the use of an additional DNA barcode or a multi-locus metabarcoding approach may be very efficient (Keller et al. 2015, Richardson et al. 2015b). Parducci et al. (2013) argued that metabarcoding and microscope-based methods can be considered as complementary in

revealing different pollen taxa. Our results show that metabarcoding is a very useful method for taxonomic assignment of pollen grains retrieved from aerobiological samples, but also that currently it cannot entirely substitute classical microscopy techniques, particularly in the case of gravimetric samples. This is where more research is needed for attaining higher resolution of the molecular method. DNA metabarcoding of airborne pollen can therefore be efficiently used for practical applications, such as assessment of air biodiversity. The metabarcoding approach provides an added value to classical analysis, by validating the taxonomic assignment and improving its resolution. In case of large-scale biodiversity studies, it could be used as a rapid tool for the taxonomic identification of pollen. Provided that protocols are customized and well-defined, the use of more automated methods and the simultaneous handling of a large number of samples can significantly increase, both quantitatively and qualitatively our ability to analyze airborne pollen.

Chapter F. Application of molecular analysis and comparisons with microscopy

F.1 Introduction

In this part of the study, following the optimized protocols described in Chapter E, we use pollen DNA metabarcoding combined with NGS to examine how closely the vegetation of different habitat types is reflected in the local pollen spectrum. More specifically, we examine the spatio-temporal patterns of airborne pollen and, hence, biodiversity and assess how well the airborne pollen reflects vegetation and phenological differences associated with time (sampling periods) and space (different habitat types and altitudes). To further evaluate the method's capacity to capture the pollen patterns and, hence, its potential to be used for monitoring vegetation changes, we use in parallel, for a same set of data, the classical microscopic method. The study was conducted in six habitat types, along the elevation gradient of the eastern Italian Alps, most of them within the National Park of Paneveggio – Pale di San Martino.

F.2 Methodology

F.2.1 Sample processing and microscopic analysis

Preliminary observations for this part of the study had been conducted during 2014. Assessment of the data collected during this preparatory phase allowed us to plan properly the phases of the experiment and have an idea of the taxa to expect. These data were analyzed only with the classical microscopic method.

Pollen sampling during the main experimental period was conducted as described in Chapter C for the periods October 2014-March 2015, March-July 2015 and July-October 2015. Solutions from the Tauber traps were filtered and processed following the protocol

described in Chapter D. The obtained pellets from the traps in each sampling point were the starting material for microscopic and molecular analysis. They are considered as replicates, since they derived from exactly the same area and after following the same sampling and processing methodology.

For the microscopic analysis, we prepared slides using a small aliquot of the pellet colored with basic fuchsine. Morphological pollen analysis was performed using an optical microscope (Leitz Diaplan, Ernst Leitz Wetzlar, Wetzlar, Germany) under 400x magnification. For the identification, we used Bucher et al. (2004). Approximately 400 pollen grains were analyzed in each sample. From this count, we calculated the absolute counts based on the counting marker we used and then we divided to the surface of the Tauber orifice. The count is finally expressed in pollen grains per unit area m² per day (pollen influx). The formula we used to find absolute counts (Faegri & Iversen 1989) is the following:

$$Total\ pollen = \frac{Pollen\ counted \ x\ Total\ number\ of\ markers}{Number\ of\ markers\ counted}$$

F.2.2 Vegetation and Aerobiological Data

For vegetation data, we used the plant checklist of Paneveggio Park and the Festi & Prosser (2000) description of the park's vegetation. We also made use of aerobiological data from the monitoring station in Paneveggio, located in Villa Wellsberg, the visitor's centre of the Park (Val Canali, 46°11′57.3"N 11°52′06.3"E, 1050 m). The station is equipped with a Lanzoni volumetric sampler (VPSS, Lanzoni, Bologna, Italy). Pollen results are obtained by use of classical microscopic analysis.

F.2.3 Molecular analysis

From the initial pollen pellet, an aliquot of 200 µl was transferred in a new extraction tube. We included a sample of a known species (*Corylus avellana*) as positive control and we had 19 negative controls (without pollen). To decrease lab contaminants, the tubes were

transferred in a non-invasive DNA extraction room (separated from PCR or post-PCR rooms), where the lysis step and the preparation of the extraction plates for Kingfisher were performed in a specialized safety cabinet. The safety cabinet was regularly UV-decontaminated and cleaned with bleach, while all materials were sterilized. For the lysis step, one sterilized steel bead, lysis buffer and RNase were added in each tube, following the manufacturer's protocol, and the tubes were covered with parafilm. A high-energy agitation (Retsch MM200 mixer mill) was applied (30 Hz for 1 min, two steps) to mechanically disrupt cell walls. The final product was transferred in an extraction plate, where the rest of the reagents were added. For the negative controls, five wells of the plate were filled with only the post-lysis reagents. The extracted DNA was eluted in 100 μl.

Pollen DNA was amplified with the universal chloroplast c-A49325 and h-B49466 *trnL* primers [5'-CGAAATCGGTAGACGCTACG-3' and 5'-CCATTGAGTCTCTGCACCTATC-3'] produced by Sigma-Aldrich (Milan, Italy) (Taberlet et al. 2007). We followed the Go Taq protocol (Promega, Madison, WI, USA) in three separate reactions for each sample, trying to maximize the chance of detecting all species in our pollen pellets (Bell et al. 2017, Sickel et al. 2015). The DNA amplification was carried out in a final volume of 50 μl using 5 μl of extracted DNA. The amplification mixture contained 1.25 U GoTaq Polymerase, 1x of GoTaq Flexi Buffer, 3 mM of MgCl2, 0.2 mM of dNTPs and 0.2 μM of each primer. All PCR amplifications were carried out on a Veriti 96 well thermal cycler (Applied Biosystems, Foster City, CA, USA) with the following program: 2 min at 95°C and 40 cycles of 15 s at 95°C, 15 s at 52°C and 30 s at 72°C, followed by 5 min at 72°C. The PCR products were electrophorized in Qiaxcel (Qiagen, GmbH, hilden, Germany). In case of no amplification, we diluted the samples (usually 1:20). In a post-PCR room, the three PCR reaction products were pooled together in equal amounts. They were further gel purified using the MinElute Gel Purification kit (Qiagen) and eluted in 53 μl. The final PCR product was quantified with Qubit 2.0 Fluorometer (Life Technologies, Thermo Fisher Scientific). The protocol for the preparation of the PCR mix and the PCR conditions in the thermocycler are summarized in Tables F.1 and F.2 respectively.

Around 100 ng of the purified PCR product were used for library preparation. A total of 54 independent libraries were generated using TruSeq DNA sample preparation kit V2 (Illumina Inc., San Diego, CA, USA), pooled in equimolar ratio and sequenced using MiSeq Reagent Kit v3 in Illumina MiSeq platform.

Table F.1 Protocol for the preparation of the PCR

Πίνακας F.1 Πρωτόκολλο για την προετοιμασία της PCR

Reagent	Start concentration	Volume (μl)	Final concentration
Water (bidistilled)		25.75	
GoTaq Flexi Buffer	5x	10	1x
MgCl$_2$	25 mM	6	3 mM
dNTPs	10 mM	1	0.2 mM
Primer Forward	10 μM	1	0.2 μM
Primer Reverse	10 μM	1	0.2 μM
GoTaq polymerase	5 U/μl	0.25	1.25 U
Template DNA		5	
Total		50	

Table F.2 PCR conditions in the thermocycler

Πίνακας F.2 Συνθήκες PCR στο θερμοκυκλοποιητή

Step	Cycles	Temperature(°C)	Time
Initial Denaturation	1	95	2 min
Denaturation	40	95	15 sec
Annealing	40	52	15 sec
Extension	40	72	30 sec
Final Extension	1	72	5 min
Soak	1	4	indefinite

F.2.4 Bioinformatics

Raw Illumina reads were paired and pre-processed using VSEARCH v2.5.0 'merge pairs' algorithm (Rognes et al. 2016). Reads were filtered out if ambiguous bases were detected and if the read lengths exceeded 100 bp. Furthermore, an expected error (=1) was used as an indicator of read accuracy. Pollen OTUs were obtained using 'cluster_fast' algorithm with a 97% sequence identity and a depth of at least 100x reads for each cluster. The centroid of the resulting cluster was chosen as the representative sequence for the taxonomic assignment step. In order to decrease the false positive rate in the sequence population, a chimera detection analysis was performed on the obtained reference sequences. Since there is no reference database for trnL gene for chimera detection, we used 'uchime_denovo' algorithm that carries out a *de novo* analysis without a reference.

Taxonomic assignments were performed by BLAST 2.4.0+ software (Camacho et al. 2009). Reference OTU sequences were aligned against our local reference database, constructed as described in Chapter D to include the most widespread anemophilous taxa of the study area (Trentino). The e-value parameter was set to a maximum value of 1e-40, above which the assignment was not considered valid. We also asked for sequence similarity and query coverage >95%. When the top score corresponded to only one species, the respective sequence was assigned at the species level. When the top score corresponded to multiple species or multiple genera, the sequence was assigned at the genus or family level, respectively (de Vere et al. 2017). An additional comparison with the non-redundant nucleotide NCBI database was performed with the same parameters in order to check the OTUs that were not assigned by our reference database.

F.2.5 Statistical analysis

Data of taxonomic assessment were analyzed with R (version 3.3.2; R core team 2016) and the R package 'vegan' (Oksanen et al. 2012).

We applied Generalized Linear Models (GLM; Gaussian error distribution, identity link function) to detect significant differences among the abundance of reads of the main

contributing pollen taxa (dependent continuous variable) and the sampling period and habitat (independent categorical variables) for the NGS dataset. We also applied GLM for the main period (March – July 2015) for the microscopic dataset to detect significant differences among pollen counts and habitat.

Alpha diversity, representing species richness, was calculated for each sample as number of identified taxa. Then, we formulated Generalized Linear Models (GLM; Poisson error distribution, log link function) to examine whether alpha diversity (dependent continuous variable) is affected by the sampling period and habitat (independent categorical variables).

As a measure of beta diversity indicating how species composition varies between samples, we used the Jaccard index, which takes into consideration presence/absence of species and estimates dissimilarity values between two samples. Patterns of beta diversity were detected by pairwise comparisons of non-metric multidimensional scaling (NMDS) ordinations. Then, we applied permutational multivariate analysis of variance (PERMANOVA) to estimate the significance of the categorical variables (habitat, sampling period) to the ordination (R 'vegan' function 'adonis'). With the variable/s found significant, we formulated Generalized Linear Models (GLM; Gaussian error distribution, identity link function) to examine how they affect beta diversity.

To compare the results of the two methods, we used the relative abundance of pollen counts and the relative abundance of sequence reads, respectively.

To investigate if the pollen influx changes between different habitats, we applied a one-way ANOVA.
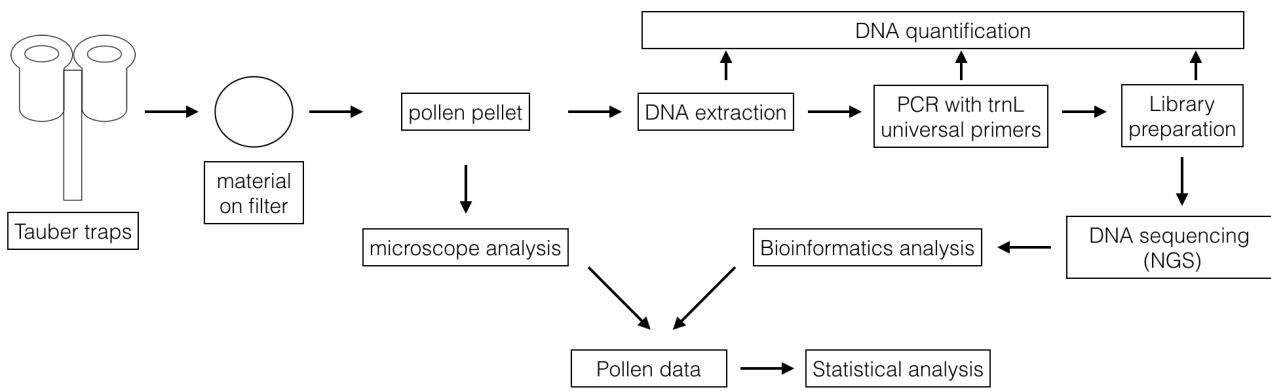
Figure F.1 Scheme of the general methodology followed in the study.

Σχήμα **F.1** *Σχηματική απεικόνιση της μεθοδολογίας που ακολουθήθηκε στην εργασία.*

F.3 Results

F.3.1 Pollen influx

The daily pollen influx, representing pollen grains m^{-2}, was calculated for the microscope data. The habitat did not have a significant effect. The highest value of pollen influx was found in a sample of *Fagus* habitat (126 pollen grains m^{-2}) (Figure F.2) and the taxon with the highest pollen influx was *Pinus* with 63 pollen grains m^{-2} in the same sample.
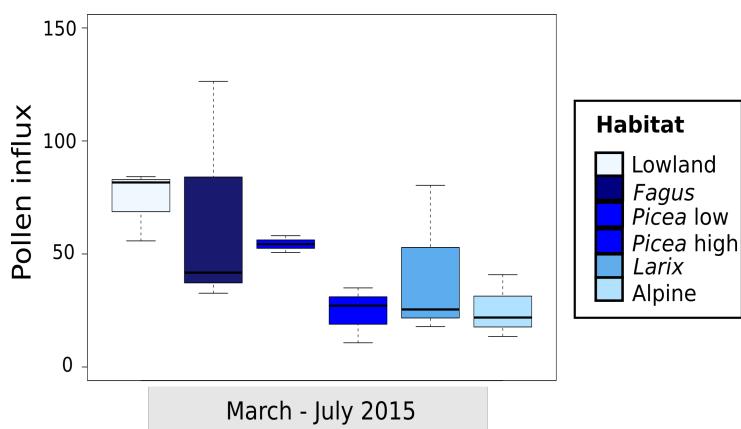
Figure F.2 Pollen influx for all different habitats within the period of March - July 2015.

Σχήμα **F.2** Εισροή γύρης για τους διαφορετικούς οικοτόπους κατά την περίοδο Μαρτίου - Ιουλίου 2015.

F.3.2 Quantitative description of NGS dataset

Our analysis gave 12,007,712 sequence reads, on average 222,365±41,954 (sd) reads per sample. Twenty OTUs, corresponding to 7.2% of the total number, were excluded from the analysis because they belonged to taxa irrelevant to our work (mainly to mosses and ferns during the periods October 2014-March 2015 and July-October 2015). The remaining 11,137,178 reads corresponding to 140 OTUs were assigned taxonomically to 32 families and 55 genera (or groups of genera) (Table F.3) or they remained unidentified (1.8%) either because they gave low identity and/or query coverage or they did not give any blast result, even when compared to the complete 'Nucleotide' Genbank database. More specifically, 37 taxa could be identified to species level, 18 taxa to genus level and 7 taxa only to family level (Appendix 5). The lowest numbers of sequence reads and of identified taxa correspond to the sampling period July-October 2015 (Table F.3).

Of the identified taxa from the NGS dataset, 37 are woody species (trees and shrubs) and 25 herbaceaous, of which 10 are graminoids (Appendix 5). Thirteen of these taxa (21%) are not present in the plant checklist of the park (Festi & Prosser 2000) and contributed with 1.3% to the total number of the sequence reads (Appendix 5). There are 13 main pollen taxa contributing at least 0.5 % to the total number of the sequence reads (Appendix 5). These are *Pinus* (36.8%), *Larix decidua* (14.5%), *Cedrus* (12.4%), *Picea* (11.6%), *Abies* (5.4%), *Corylus/Ostrya/Carpinus* (5%), *Alnus viridis* (2.9%), *Urtica dioica* (2.8%), *Juniperus communis* (0.7%), *Taxus baccata* (0.6%), *Chenopodium album* (0.6%), *Festuca/Trisetum/Lolium* (0.5%) and *Cupressus sempervirens* (0.5%). In the sequencing results, the positive control was indeed pollen of *Corylus avellana* as it should. From our negative controls, 17% were found contaminated, but at very low concentrations. One sample of these was sequenced, as its level of contamination allowed for library preparation and sequencing. Contamination was found to be from *Quercus*.

The pollen spectrum per sampling period is shown in Figure F.3. Detailed data on the pollen taxa identified and their quantitative contribution in the samples of the three periods are presented in Appendix 5. The main pollen taxa per period (contributing with at least 0.5% in each one) are *Larix decidua*, *Pinus*, *Corylus/Ostrya/Carpinus*, *Picea*, *Cedrus*,

Alnus viridis, Cupressaceae (*Juniperus communis, Cupressus sempervirens, Juniperus sabina*), *Taxus baccata, Ulmus glabra,* and *Fraxinus/Olea/Syringa* for the period October 2014-March 2015; *Pinus, Picea, Abies, Larix decidua* and *Festuca/Trisetum/Lolium* for March-July 2015; and *Cedrus, Pinus, Picea,* Urticaceae (*Urtica dioica* and *Parietaria judaica*) *Corylus/Ostrya/Carpinus, Alnus viridis, Larix decidua, Chenopodium album*, and Poaceae *(Calamagrostis epigeos, Festuca/ Trisetum/Lolium, Avenella flexuosa)* for July - October 2015 (Figure F.3). The pollen spectrum is overwhelmingly simple in the sampling period March-July 2015.

Table F.3 Quantitative data for the sequences detected and the pollen contributing taxa that were identified at the different sampling periods.

Πίνακας F.3 Ποσοτικά δεδομένα για τις ακολουθίες που βρέθηκαν και τα τάξα που αναγνωρίστηκαν στις διάφορες περιόδους δειγματοληψίας.

Sampling period	No. of sequences	No. of identified genera or group of genera (species)	No. of identified families
October 2014-March 2015	3287283	50 (33)	29
March-July 2015	4166877	51 (34)	31
July-October 2015	3683018	34 (23)	22
October 2014-October 2015	11137178	55 (37)	32

F.3.3 Spatio-temporal pollen patterns

High pollen concentrations of the main pollen NGS taxa coincide with their pollen seasons, as they were defined after the data from the aerobiological station of the Park (Figure F.4). Exception to that are *Corylus/Ostrya/Carpinus* and *Alnus viridis*, the pollen of which was found in our samples at relatively high concentrations both within and outside their pollen seasons. Also, for *Chenopodium album* and *Urtica dioica*, pollen was detected only in the period July-October 2015, although their pollen season started within the period March-July 2015 (Figure F.4). Significant differences ($p < 0.05$) in mean pollen

occurrence among sampling periods were found for eight of the 13 main pollen taxa. More specifically, for *Cupressus sempervirens, Juniperus communis* and *Larix decidua* significantly higher concentrations were found in October 2014-March 2015; for *Pinus* and *Abies* in March-July 2015; and for *U. dioica*, *Cedrus* and *C. album* in July-October 2015.

The majority of the main pollen taxa (11 of 13 taxa) have representatives at least in one of the habitat types examined (Appendix 6), except for *Cedrus* and *C. sempervirens* (Figure F.4) that do not occur within the Park; notably, the latter is found at all altitudes. Regardless of concentration, *Pinus, L. decidua, Cedrus, Picea* and *Abies* were found in all samples from all habitats (except for one sample from one habitat for *Abies*) and sampling seasons (Appendix 5).

Applying GLM only for the main period March-July 2015, we found that habitat affected significantly (p<0.05) the abundance of *Festuca/Trisetum/Lolium*, *A. viridis*, *L. decidua* and *Taxus baccata* for the NGS dataset. Similarly, it significantly affected *Alnus* and *Larix decidua* abundance for the microscopic dataset. As *Festuca/Trisetum/Lolium* and *T. baccata* were only part of the pollen taxa of grasses and Cupressaceae-Taxaceae, respectively, that were identifiable by the microscopic method only at the higher taxonomic level, no comparison could be made for them. Using the microscopic method, a significant effect of habitat was found also on *Cedrus* abundance, which appeared only in two samples with very low counts (4 in total).

After both NGS and microscope, pollen occurrence of *L. decidua* had a peak in the *Larix* habitat, *Picea* was detected more in *Picea* high and *Larix* habitats and the graminoids were detected mainly in the lowest and the highest altitudes, where grasslands occur (Figure F.3). It is noteworthy that *Fagus* did not appear in the sequencing results, neither at the pollen monitoring data of the same year.

(a)

(b)

Taxa

■ Unidentified	■ *Taxus baccata*
■ Other taxa	■ *Chenopodium album* (Chenopodiaceae)
■ *Pinus*	■ *Festuca/Trisetum/Lolium* (Poaceae)
■ *Larix decidua*	■ *Cupressus sempervirens*
■ *Cedrus*	■ *Fraxinus/Olea/Syringa*
■ *Picea*	■ *Betula*
■ *Abies*	■ *Quercus*
■ *Corylus/Ostrya/Carpinus* (*Ostrya*)	■ Juncaceae
■ *Alnus viridis* (*Alnus*)	■ Asteraceae
■ *Urtica dioica* (Urticaceae)	■ *Plantago*
■ *Juniperus communis* (Cupressaceae)	

Figure F.3 Pollen spectrum **(a)** for the period March-July 2015, as derived by microscopy, and **(b)** for all three periods of the study, as derived by NGS. Given separately are the main taxa representing ≥ 0.5% of the total pollen count, on a yearly basis for NGS, and for the main period for microscopic data. The other taxa are grouped under 'other taxa', if identified, and if not, under 'unidentified'. If the level of taxonomic identification differed between methods, given in parenthesis is the level after the microscopic method. For the full pollen spectrum, see Appendix 5. The x-axis corresponds to habitat type (three replicates for each), from low to high altitudes for each period; 1=Lowland, 2=*Fagus* habitat, 3=*Picea* habitat-low, 4=*Picea* habitat-high, 5=*Larix* habitat, 6=Alpine. The full names of the corresponding habitats are given in Table C.2.

Σχήμα F.3 Φάσματα γύρης **(a)** για την περίοδο Μαρτίου-Ιουλίου 2015, όπως βρέθηκαν με το μικροσκόπιο, **(b)** για τις τρεις δειγματοληπτικές περιόδους, όπως βρέθηκαν με την μέθοδο NGS. Ξεχωριστά δίνονται τα κύρια τάξα που αντιπροσωπεύουν ≥0.5% της συνολικής γύρης, σε ετήσια βάση για την NGS και και για την κύρια περίοδο για την μικροσκοπική μέθοδο. Τα υπόλοιπα τάξα ομαδοποιούνται ως 'other taxa', αν αναγνωρίστηκαν, και αν όχι ως 'unidentified'. Αν το επίπεδο της ταξινομικής αναγνώρισης διέφερε μεταξύ μεθόδων, δίνεται σε παρένθεση το επίπεδο το οποίο μπορεί να αναγνωριστεί με τη μέθοδο της μικροσκοπίας. Τα πλήρη φάσματα γύρης δίνονται στο Παράρτημα 5. Ο χ άξονας αντιστοιχεί στον τύπο οικοτόπου (3 δείγματα για καθένα), από τα χαμηλά στα μεγάλα υψόμετρα για κάθε περίοδο; 1=χαμηλά υψόμετρα, 2=οικότοπος *Fagus*, 3=οικότοπος *Picea* χαμηλά, 4=οικότοπος *Picea* ψηλά, 5=οικότοπος *Larix*, 6=αλπικοί οικότοποι. Τα πλήρη ονόματα των οικοτόπων δίνονται στον Πίνακα C.2.

Figure F.4 Pollen occurrence (%) of the taxa representing ≥ 0.5% of the annual pollen count per sampling period (upper left, grey part of each graph) and per habitat (upper right, coloured part of each graph) after NGS. For comparative purposes, apart from these data, given are also below them aerobiological and vegetation data indicating when each taxon is represented in the airborne pollen (yellow) and where it occurs in the vegetation (green). The full names of the habitat types are given in Table C.2.

Σχήμα F.4 Παρουσία γύρης (%) των ταξα που αντιπροσωπεύουν ≥ 0.5% της ετήσιας γύρης ανά χρονική περίοδο (πάνω αριστερά, γκρί κομμάτι κάθε γραφήματος) και ανά οικότοπο (πάνω δεξιά, με χρώμα κομμάτι κάθε γραφήματος) μετά από NGS. Για λόγους σύγκρισης, εκτός από αυτά τα δεδομένα, δίνονται επίσης από κάτω δεδομένα αεροβιολογίας και βλάστησης που δείχνουν πότε κάθε τάξο αντιπροσωπεύεται στην αερομεταφερόμενη γύρη (κίτρινο) και που υπάρχει στη βλάστηση (πράσινο). Τα ολόκληρα ονόματα των οικοτόπων δίνονται στον Πίνακα C.2

F.3.4 NGS vs. microscopy

For the period March-July 2015, we compared results taken by use of NGS and the microscopic method. With NGS, 62 taxa were detected, whereas 50 with the microscope (Figure F.7b, Appendix 5). With both methods, we found 39 families to be represented in the pollen spectrum, of which 25 were common to the two datasets. Within these common families, NGS could distinguish 51 genera or groups of genera compared to 26 of the microscope method; the common genera were only 14 (Figure F.5). In general, families absent in the outputs of any of the two methods (8 absent from the NGS and 6 absent from the microscope outputs) were scarcely represented, occurring only in one or two samples (rare families). However, this does not hold for Cyperaceae and Polygonaceae, which were detected only with the classical microscopic method.

Comparing the contribution of each family in the pollen spectra, as derived by the two methods (Figure F.3, Appendix 5), we observe that the microscopic method detected systematically more pollen of Poaceae, Betulaceae, Corylaceae and Oleaceae than NGS, whereas the latter detected more pollen of Pinaceae. Nevertheless, *Pinus* was the most abundantly represented taxon after both the number of reads for NGS and the pollen counts for the microscope (Appendix 5). Although the ranking of the other taxa differed between the two methods, eight of the next ten most abundant taxa were the same in either case. Asteraceae and Ericaceae after NGS were replaced by Corylaceae and Oleaceae after the microscope.

We should note that a considerable part of pollen counts (12%) remained unidentified with the microscopic method. They could not be attributed to any taxon because of unclear features resulting most of the times from the degradation of the pollen wall structure or because they could not be assigned with certainty to any of the plants known to occur in the study area.
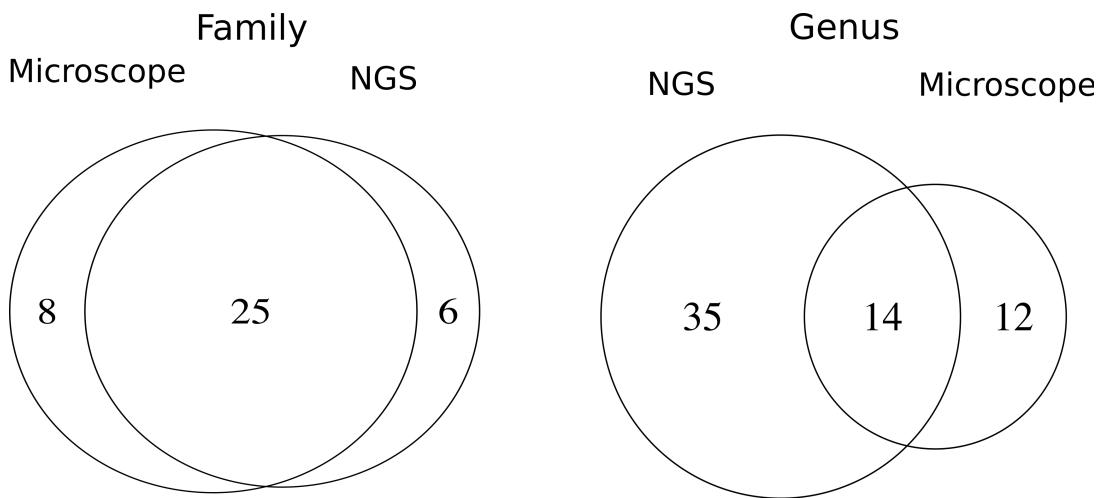
Figure F.5 Venn diagrams that show the number of taxa found by NGS and Microscope and their commonly found taxa during March - July 2015

Σχήμα F.5 Διαγράμματα Venn που δείχνουν τον αριθμό taxa και κοινών taxa που βρέθηκαν με NGS και μικροσκόπιο κατά την περίοδο Μαρτίου - Ιουλίου 2015

F.3.5 Biodiversity measures

For the NGS dataset, the sampling period influenced significantly alpha diversity, with that of October 2014-March 2015 and March-July 2015 differing significantly from July-October 2015 (GLM, p<0.001, R²=0.99) (Figure 6a). The period of October 2014-March 2015 did not differ from that of March-July 2015 regarding alpha diversity, but it was double in duration. Instead, the period of July-October 2015 although of the same sampling duration with March-July 2015,it had half number of identified taxa.

To be able to compare the periods of different sampling duration, the number of taxa found in each sample was divided by the corresponding number of days of the sampling period. This standardization on a per-sampling day basis gave us an estimate of the pollen taxa turnover in the different periods (Figure 7).

The sampling period also influenced beta diversity, more specifically the Jaccard index (GLM, p<0.001, R²=0.43 and PERMANOVA, pseudo-F= 14.2, p<0.001, R²=0.35). The NMDS ordination groups together samples of the same period (Figure 8a). Beta diversity is lower within periods than among periods. From the values of the Jaccard index (Figure

8b), it results that the highest dissimilarity among samples is observed in the period July-October 2015. For the other two periods, samples are quite homogeneous, although representing different habitats. Comparing the number of samples (%), in which each taxon was present during the three periods (Appendix 5), Cupressaceae taxa (*Cupressus arizonica*, *Thuja orientalis*, *Taxodium distichum*), *Taxus baccata a*nd *Alnus* determine the profile of the period October 2014-March 2015, Poaceae taxa and *Ambrosia*/*Taraxacum*/others from Asteraceae that of March-July 2015, whereas *Chenopodium album* that of July-October 2015.

Habitat did not significantly affect neither alpha nor beta diversity, after either of the two methods.
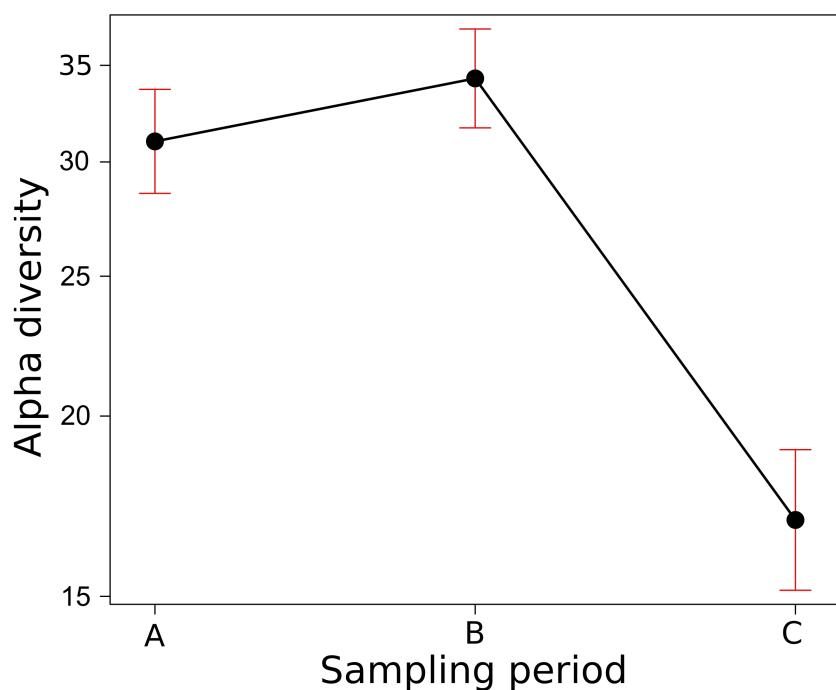
Figure F.6 Alpha diversity as number of identified taxa (mean ± standard error) for each sampling period. A = October 2014-March 2015, B = March-July 2015, C = July-October 2015.

Σχήμα F.6 Άλφα ποικιλότητα ως αριθμός των τάξα που αναγνωρίστηκαν (μέσος όρος ± τυπικό σφάλμα) ανα περίοδο δειγματοληψίας. Α = Οκτώβριος 2014-Μάρτιος 2015, Β = Μάρτιος-Ιούλιος 2015, C = Ιούλιος-Οκτώβριος 2015

Figure F.7 Number of species per habitat and sampling period divided by the number of days each one lasted, after NGS (from low to high altitudes). The full names of the habitat types are given in Table C.2

Σχήμα F.7 Αριθμός ειδών ανά οικότοπο και δειγματοληπτική περίοδο διαιρεμένος με τον αριθμό ημερών της εκάστοτε δειγματοληπτικής περιόδου, όπως προσδιορίστηκαν με την μέθοδο NGS (από τα χαμηλά στα μεγαλα υψόμετρα). Τα πλήρη ονόματα των οικοτόπων δίνονται στον Πίνακα C.2.
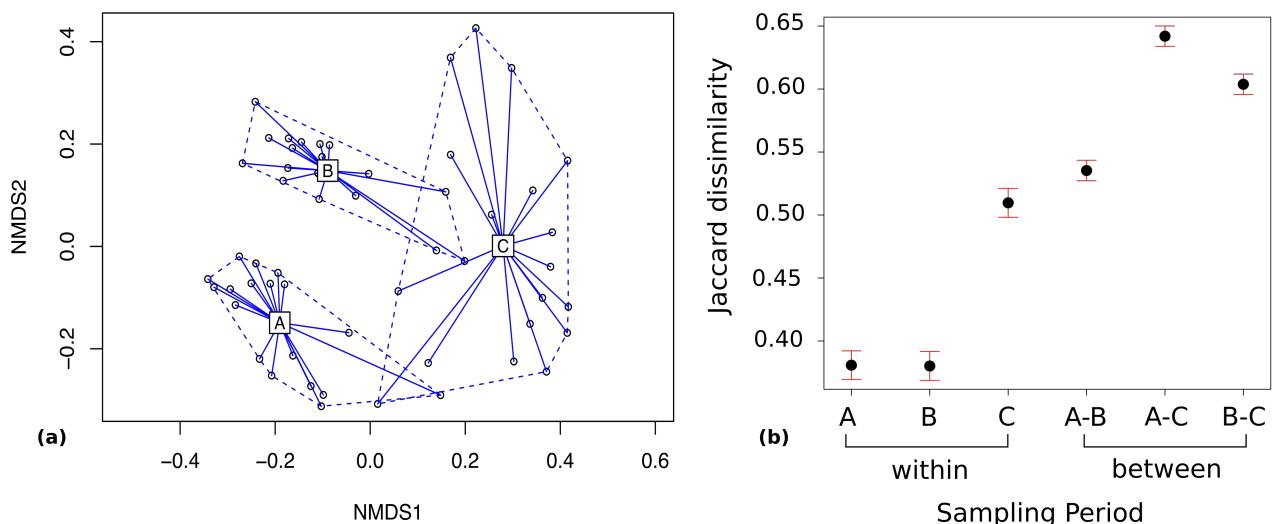
Figure F.8 Beta diversity estimates after NGS: **(a)** Non-metric multidimensional scaling (NMDS) using Jaccard index, with samples grouped according to the sampling period, **(b)** effect plot (mean ± standard error) of within and between sampling periods dissimilarity estimated by the Jaccard index. A = October 2014-March 2015, B = March-July 2015, C = July-October 2015.

Σχήμα F.8 Η β–ποικιλότητα, όπως εκτιμήθηκε με την NGS: (a) ταξιθέτηση με τη μέθοδο NMDS, χρησιμοποιώντας το δείκτη Jaccard, με τα δείγματα ομαδοποιημένα κατά περίοδο δειγματοληψίας, (b) διάγραμμα επιδράσεων (μέσος όρος ± τυπικό σφάλμα) για την εντός και μεταξύ περιόδων ανομοιότητα, όπως εκτιμήθηκε με το δείκτη Jaccard. A = Οκτώβριος 2014-Μάρτιος 2015, B = Μάρτιος-Ιούλιος 2015, C = Ιούλιος-Οκτώβριος 2015.

F.4 Discussion

Results of the microscopic and metabarcoding methods were in general quite similar. Nevertheless, there were some notable differences. This is the case with Cyperaceae and Polygonaceae that did not feature in the metabarcoding results although they were present with considerable abundance in the microscope dataset. This may be due to an inherent lack of universality of the primers, resulting in PCR priming sites mismatching (Keller et al. 2015). For the other families that appeared in the results of one of the two methods (Appendix 5), pollen abundance was very low. Within the common families, the taxonomic information resulting from the metabarcoding method is richer (more taxa) and of higher resolution (results even at the species level) compared to microscope results, what is in agreement with previous studies (Bell et al. 2017, de Vere et al. 2016, Hawkins et al. 2015, Jørgensen et al. 2012, Keller et al. 2015, Laha et al. 2017, Pornon et al. 2017, Smart et al. 2016).

Apart from the qualitative (taxonomic) differences of the pollen spectrum, there were also quantitative (abundance) differences. Compared to microscopic results, Pinaceae seem to be overrepresented, whereas Poaceae, Betulaceae, Corylaceae and Oleaceae underrepresented in the NGS pollen spectrum. Similar discrepancies were reported earlier when either nuclear or chloroplast markers were used to analyze mixed pollen samples (Hawkins et al. 2015, Kraaijeveld et al. 2015, Richardson et al. 2015a, Richardson et al. 2015b, Keller et al. 2015). Because of the quantitative issues of the method, not yet fully settled, we chose the presence/absence-based Jaccard index for the diversity estimations rather than abundance-based indices (Zinger et al. 2012).

We found the sampling period to influence significantly both alpha and beta pollen diversity. Among sampling periods, alpha diversity maximizes in March-July 2015. This is in agreement with aerobiological data showing that most taxa have their pollen seasons within this period (Cristofori et al. 2010, Appendix 4). For beta diversity, the sampling period is clearly more important than the habitat type in creating distinct sets of pollen taxa. This is related to the fact that although local vegetation is reflected in the local pollen

spectrum, there is also pollen arriving from elsewhere. But different species are in flower at the different sampling periods and, therefore, the sample homogeneity is low. Núñez et al. (2017) also detected remarkable differences in the NGS pollen spectrum between winter and summer samples.

In several cases, pollen was collected from sites, where the producing taxa were absent. Obviously, this indicates transfer from elsewhere (Mohanty et al. 2017), possibly, from inside the Park (*Taxus baccata*, *Chenopodium album*, *Corylus/Ostrya/Carpinus*, *Abies* and *Pinus*) or, certainly, outside it (*Cedrus, Cupressus sempervirens*). In particular, Zorer et al. (2014) reported that Trentino is an area where *C. sempervirens* has been introduced long ago and it is distributed within the altitudinal range of 66 m to 985 m, while *Cedrus* is a tree found in the urban parks and gardens of the area (http://www.comune.trento.it). Given the distribution of C. *sempervirens* in the area of Trentino, we can estimate a distance of approximately 20 km, on a straight line, as that the shortest distance of C. *sempervirens* pollen transfer to our traps.

Furthermore, all Pinaceae taxa (*Pinus, Larix, Cedrus, Picea, Abies*) have a ubiquitous presence; they are found in the samples of all sampling seasons and all (or almost) habitats. This cannot be explained only in terms of transfer from elsewhere because these taxa are represented in the pollen spectrum beyond the pollen seasons of their members. This is particularly true for Pinaceae that have an overwhelming contribution to the pollen spectrum of Paneveggio making 81% of the total pollen count. As a result of this huge production, their pollen may remain on the ground for long contaminating samples in periods beyond the time when it is produced and released. Especially for *Pinus,* which was the most abundant pollen taxon after either method, its dominance is related to the fact that the very light pollen that it produces gets easily transported (Pidek et al. 2010), easier than that of other Pinaceae members. Such a transport was confirmed by Van der Knaap (2001); using Tauber traps, he found higher amounts of *Pinus* pollen above the treeline than in lower mountainous areas, what indicates upslope transport by wind currents.

It is notable that the pollen of spruce, which has an overwhelming participation in the forests of Paneveggio (~85%), is only fourth in rank contributing with 11.6% of the

total pollen (8% with the microscopic method). This under-representation of *Picea* pollen in the pollen spectrum has been also reported in the past (Pidek et a. 2010). The reasons have been sought in relation to its big and heavy grains (Pidek et a. 2010).

Most of the taxa detected are represented in the vegetation locally and appear at sampling times which correspond to their pollen seasons, as defined after the aerobiological data. For some taxa that are so far identified only collectively with the classical approaches, as are grasses, the new method proves very important for both diagnostic and treatment purposes (Ghitarrini et al. 2017) as it can identify pollen taxa at far lower levels than that of the family. However, even for grasses, it is not wise to abandon the microscopic method because of the rather low quantitative accuracy of the molecular method. As the study area is a protected area, NGS could be also applied to reveal possible invasion of alien species.

With NGS, our ability to analyze pollen datasets increases considerably in qualitative terms, but more research is needed to achieve better resolution in quantitative terms. Providing high resolution taxonomic results, the method can be used for biodiversity assessments and floral surveys or to monitor vegetation changes, particularly those expressed in species composition rather than in species abundance. However, we should bear in mind its inability to detect representatives of Polygonaceae and Cyperaceae. On the basis of our results and previous reports, we can argue that the metabarcoding and the microscopic methods have each their weak and strong points and they should be applied in a complementary way, at least until the quantitative and qualitative issues associated with metabarcoding are adequately addressed.

Chapter G. Conclusions

The major conclusions from our research attempting to develop optimal protocols for the study of pollen DNA from environmental samples and applying these protocols for the study of plant biodiversity on a mountainous area (Paneveggio National Park, Italy) and for the detection of spatial and temporal pollen patterns are the following:

- Regarding the protocol, recommended are steel beads for the disruption of the pollen walls, more than two washings for cleaning the pollen pellets, and Nucleomag kit for DNA isolation.

- Application of the optimized protocol along with traditional cloning and sequencing techniques to environmental pollen, collected by volumetric (Burkard, Lanzoni) and gravimetric samplers (Tauber), had a taxonomic efficiency of 75% and 37%, respectively, taking as criterion the number of taxa detected with the molecular method compared to those with the microscope. The efficiency of the molecular method rose to 70% with the application of next generation sequencing.

- Pollen temporal patterns could be distinguished: the sampling periods differed from each other regarding diversity attributes. High pollen concentrations of the main pollen taxa coincide with their pollen seasons, as defined after data from the aerobiological station of the Park.

- Pollen spatial patterns could not be clearly distinguished: the habitat did not have a significant effect on diversity attributes.

- There is input of pollen from outside the park. For 21% of the pollen taxa detected, there were no representatives in the park's vegetation; *Cedrus* and *Cupressus* pollen, in particular, had a considerable contribution.

- *Pinus* is the dominant pollen taxon accounting for 37% of the annual sum. Nevertheless, its contribution to the park's vegetation is lower than that of the dominant *Picea* (85%), the pollen of which contributes only 12% to the annual sum.

This discrepancy was observed not only with the NGS method of analysis but also with the microscopic method and was also detected in the aerobiological data (Lanzoni sampler).

- Comparison of NGS data to those of the classical microscopic method showed similarity to a considerable degree. Nevertheless, a number of quantitative but also qualitative differences were observed.

- Polygonaceae and Cyperaceae pollen was not detected with the NGS method, although its abundance in the microscopic dataset was not negligible (0.4% and 0.6%, respectively).

- Pinaceae contributed more, whereas Poaceae, Betulaceae, Corylaceae and Oleaceae contributed less in the NGS dataset than in the microscopic dataset.

- NGS is an efficient method for biodiversity assessment and monitoring. However, taxa at low concentrations in environmental samples may not be detected.

- Till a number of issues, primarily quantitative, are resolved, it is advisable that traditional and molecular methods are used in a complementary way for pollen analysis.

References

Ahn, J. H., Kim, B. Y., Song, J., & Weon, H. Y. (2012). Effects of PCR cycle number and DNA polymerase type on the 16S rRNA gene pyrosequencing analysis of bacterial communities. Journal of Microbiology, 50(6), 1071–1074. https://doi.org/10.1007/s12275-012-2642-z

Baamrane, M. A., Shehzad, W., Ouhammou, A., Abbad, A., Naimi, M., Coissac, E., et al. (2012). Assessment of the food habits of the Moroccan dorcas gazelle in M'Sabih Talaa, west central Morocco, using the trnL approach. PLoS ONE, 7(4). https://doi.org/10.1371/journal.pone.0035643

Amadei, M., Capogrossi, R., Francescato, C., Giacanelli, V., Laureti L., Lisi, A., et al. (2005). Carta della Natura e Biodiversità nelle aree naturali protette: il parco naturale Paneveggio-Pale di San Martino. APAT - Agenzia per la protezione dell'ambiente e per i servizi tecnici, Rome.

Andersen, K., Bird, K. L., Rasmussen, M., Haile, J., Breuning-Madsen, H., Kjær, K. H., et al. (2012). Meta-barcoding of "dirt" DNA from soil reflects vertebrate biodiversity. Molecular Ecology, 21(8), 1966–1979. https://doi.org/10.1111/j.1365-294X.2011.05261.x

Ando, H., Setsuko, S., Horikoshi, K., Suzuki, H., Umehara, S., Inoue-Murayama, M., et al. (2013). Diet analysis by next-generation sequencing indicates the frequent consumption of introduced plants by the critically endangered red-headed wood pigeon (Columba janthina nitens) in oceanic island habitats. Ecology and Evolution, 3(12), 4057–4069. https://doi.org/10.1002/ece3.773

Arezi, B., Xing, W., Sorge, J. A., & Hogrefe, H. H. (2003). Amplification efficiency of thermostable DNA polymerases. Analytical Biochemistry, 321(2), 226–235. https://doi.org/10.1016/S0003-2697(03)00465-2

Awad, A. H. A. (2005). Vegetation: A source of air fungal bio-contaminant. Aerobiologia 21(1), 53-61. https://doi.org/10.1007/s10453-004-5878-1

Bakker, M. G., Tu, Z. J., Bradeen, J. M., & Kinkel, L. L. (2012). Implications of Pyrosequencing Error Correction for Biological Data Interpretation. PLoS ONE, 7(8), e44357. http://doi.org/10.1371/journal.pone.0044357

Barberàn, A., Fernàndez-Guerra, A., Bohannan, B. J. M., & Casamayor, E. O. (2012). Exploration of community traits as ecological markers in microbial metagenomes. Molecular Ecology, 21(8), 1909–1917. https://doi.org/10.1111/j.1365-294X.2011.05383.x

Beaubien, E. G., & Freeland, H. J. (2000). Spring phenology trends in Alberta, Canada: links to ocean temperature. International Journal of Biometeorology, 44(2), 53–59. https://doi.org/10.1007/s004840000050

Bell, K. L., de Vere, N., Keller, A., Richardson, R. T., Gous, A., Burgess, K. S., & Brosi, B. J. (2016). Pollen DNA barcoding: current applications and future prospects. Genome, 59(9), 629–640. https://doi.org/10.1139/gen-2015-0200

Bell, K. L., Fowler, J., Burgess, K. S., Dobbs, E. K., Gruenewald, D., Lawley, B., et al. (2017). Applying Pollen DNA Metabarcoding to the Study of Plant–Pollinator Interactions. Applications in Plant Sciences, 5(6), 1600124. https://doi.org/10.3732/apps.1600124

Bellemain, E., Carlsen, T., Brochmann, C., Coissac, E., Taberlet, P., & Kauserud, H. (2010). ITS as an environmental DNA barcode for fungi: an in silico approach reveals potential PCR biases. BMC Microbiology, 10(1), 189. http://doi.org/10.1186/1471-2180-10-189

Belmonte, J., Canela, M., & Guàrdia, R. A. (2000). Comparison between categorical pollen data obtained by Hirst and Cour sampling methods. Aerobiologia, 16(2), 177–185. https://doi.org/10.1023/A:1007628214350

Bennett, K. D., & Parducci, L. (2006). DNA from pollen: Principles and potential. Holocene, 16(8), 1031–1034. https://doi.org/10.1177/0959683606069383

Benninghoff, W. S. (1991). Aerobiology and its significance to biogeography and ecology. Grana, 30(1), 9–15. https://doi.org/10.1080/00173139109427762

Bhattacharya, A., Mondal, S., & Mandal, S. (1999). Entomophilous pollen incidence with reference to atmospheric dispersal in eastern India. Aerobiologia, 15(4), 311-315. https://doi.org/10.1023/A:1007690128025

Bienert, F., De Danieli, S., Miquel, C., Coissac, E., Poillot, C., Brun, J. J., et al. (2012). Tracking earthworm communities from soil DNA. Molecular Ecology, 21(8), 2017–2030. https://doi.org/10.1111/j.1365-294X.2011.05407.x

Biesbroek, G., Sanders, E. A. M., Roeselers, G., Wang, X., Caspers, M. P. M., Trzciński, K., et al. (2012). Deep sequencing analyzes of low density microbial communities: Working at the boundary of accurate microbiota detection. PLoS ONE, 7(3), e32942. https://doi.org/10.1371/journal.pone.0032942

Blaalid, R., Carlsen, T., Kumar, S., Halvorsen, R., Ugland, K. I., Fontana, G., et al. (2012). Changes in the root-associated fungal communities along a primary succession gradient analyzed by 454 pyrosequencing. Molecular Ecology, 21(8), 1897–1908. https://doi.org/10.1111/j.1365-294X.2011.05214.x

Blaxter, M., Mann, J., Chapman, T., Thomas, F., Whitton, C., Floyd, R., et al. (2005). Defining operational taxonomic units using DNA barcode data. Philosophical Transactions of the Royal Society B: Biological Sciences, 360(1462), 1935–1943. http://doi.org/10.1098/rstb.2005.1725

Blionis, G. J., Halley, J. M., & Vokou, D. (2001). Flowering phenology of Campanula on Mt Olympos, Greece. Ecography, 24(6), 696–706. https://doi.org/10.1034/j.1600-0587.2001.240608.x

Boessenkool, S., Mcglynn, G., Epp, L. S., Taylor, D., Pimentel, M., Gizaw, A., et al. (2014). Use of ancient sedimentary DNA as a novel conservation tool for high-altitude tropical biodiversity. Conservation Biology, 28(2), 446–455. https://doi.org/10.1111/cobi.12195

Bohmann, K., Evans, A., Gilbert, M. T. P., Carvalho, G. R., Creer, S., Knapp, M., et al. (2014). Environmental DNA for wildlife biology and biodiversity monitoring. Trends in Ecology and Evolution, 29(6), 358–367. https://doi.org/10.1016/j.tree.2014.04.003

Bolick, M. R., & Vogel, S. (1992). Breaking strengths of pollen grain walls. Plant Systematics and Evolution, 181(3–4), 171–178. https://doi.org/10.1007/BF00937442

Bonton, P., Boucher, A., Thonnat, M., Tomczak, R., Hidalgo, P., Belmonte, J., et al. (2001). Colour Image in 2D and 3D Microscopy for the Automation of Pollen Rate Measurement. Image Anal. Stereol., 20(Suppl 1), 517–532. https://doi.org/10.5566/ias.v21.p25-30

Borrell, Y. J., Miralles, L., Do Huu, H., Mohammed-Geba, K., & Garcia-Vazquez, E. (2017). DNA in a bottle - Rapid metabarcoding survey for early alerts of invasive species in ports. PLoS ONE, 12(9), 1–17. https://doi.org/10.1371/journal.pone.0183347

Bozabalidis A. 2003. Botany. Morphology & Anatomy of Plants. Thessaloniki (in greek)

Bradley, N. L., Leopold, A. C., Ross, J., & Huffaker, W. (1999). Phenological changes reflect climate change in Wisconsin. Proceedings of the National Academy of Sciences, 96(17), 9701–9704. https://doi.org/10.1073/pnas.96.17.9701

Bruni, I., Galimberti, A., Caridi, L., Scaccabarozzi, D., De Mattia, F., Casiraghi, M., et al. (2015). A DNA barcoding approach to identify plant species in multiflower honey. Food Chemistry, 170, 308–315. https://doi.org/10.1016/j.foodchem.2014.08.060

Bruno, A., Sandionigi, A., Rizzi, E., Bernasconi, M., Vicario, S., Galimberti, A., et al. (2017). Exploring the under-investigated "microbial dark matter" of drinking water treatment plants. Scientific Reports, 7, 1–7. https://doi.org/10.1038/srep44350

Bucher, E., Kofler, V., Vorwohl, G. & Zieger, E. (2004). Lo spettro pollinico dei mieli dell Alto Adige. Laboratorio biologico dell'Agenzia per la protezione dell'ambiente e la tutela del lavoro, Laives

Buters, J.T.M., Thibaudon, M., Smith, M., Kennedy, R., Rantio-Lehtimäki, A., Albertini, R., et al. (2012). Release of Bet v 1 from birch pollen from 5 European countries. Results from the HIALINE study. Atmospheric Environment, 55, 496–505. https://doi.org/10.1016/j.atmosenv.2012.01.054

Cai, L., Ye, L., Tong, A. H. Y., Lok, S., & Zhang, T. (2013). Biased Diversity Metrics Revealed by Bacterial 16S Pyrotags Derived from Different Primer Sets. PLoS ONE, 8(1), 1–11. https://doi.org/10.1371/journal.pone.0053649

Calvignac-Spencer, S., Merkel, K., Kutzner, N., Kühl, H., Boesch, C., Kappeler, P. M., et al. (2013). Carrion fly-derived DNA as a tool for comprehensive and cost-effective assessment of mammalian biodiversity. Molecular Ecology, 22(4), 915–924. https://doi.org/10.1111/mec.12183

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., et al. (2009). BLAST+: Architecture and applications. BMC Bioinformatics, 10, 1–9. https://doi.org/10.1186/1471-2105-10-421

Carvalho, E., Sindt, C., Verdier, A., Galan, C., O'Donoghue, L., Parks, S., et al. (2008). Performance of the Coriolis air sampler, a high-volume aerosol-collection system for quantification of airborne spores and pollen grains. Aerobiologia, 24(4), 191–201. https://doi.org/10.1007/s10453-008-9098-y

Charalampopoulos 2010. Pollen Atmospheric Circulation and Production along the altitudinal gradient of Mt Olympus National Park. MSc Thesis, School of Biology, Aristotle University of Thessaloniki

Charalampopoulos, A., Damialis, A., Tsiripidis, I., Mavrommatis, T., Halley, J. M., & Vokou, D. (2013). Pollen production and circulation patterns along an elevation gradient in Mt Olympos (Greece) National Park. Aerobiologia, 29(4), 455–472. https://doi.org/10.1007/s10453-013-9296-0

Charalampopoulos, A., Lazarina, M., Tsiripidis, I. & Vokou, D. (2018). Quantifying the relationship between airborne pollen and vegetation in the urban environment. Aerobiologia. https://doi.org/10.1007/s10453-018-9513-y

Chuine, I., Cambon, G., & Comtois, P. (2000). Scaling phenology from the local to the regional level: Advances from species-specific phenological models. Global Change Biology, 6(8), 943–952. https://doi.org/10.1046/j.1365-2486.2000.00368.x

Cleland, E. E., Chuine, I., Menzel, A., Mooney, H. A., & Schwartz, M. D. (2007). Shifting plant phenology in response to global change. Trends in ecology & evolution, 22(7), 357-365. https://doi.org/10.1016/j.tree.2007.04.003

Coissac, E., Riaz, T., & Puillandre, N. (2012). Bioinformatic challenges for DNA metabarcoding of plants and animals. Molecular Ecology, 21(8), 1834–1847. https://doi.org/10.1111/j.1365-294X.2012.05550.x

Creer, S., Deiner, K., Frey, S., Porazinska, D., Taberlet, P., Thomas, W. K., et al. (2016). The ecologist's field guide to sequence-based identification of biodiversity. Methods in Ecology and Evolution, 7(9), 1008–1018. https://doi.org/10.1111/2041-210X.12574

Crispen, K. L., Gillespie, D. N., Weiler, E. C., Noonan, C. W., Hamilton, R. F., & Ward, T. J. (2010). A comparison of 1978 and 2006 peak pollen seasons and sampling methods in Missoula, Montana. Grana, 49(2), 128-133. https://doi.org/10.1080/00173131003587049

Cristofori, A., Cristofolini, F., & Gottardini, E. (2010). Twenty years of aerobiological monitoring in Trentino (Italy): Assessment and evaluation of airborne pollen variability. Aerobiologia, 26(3), 253–261. https://doi.org/10.1007/s10453-010-9161-3

Crouzy, B., Stella, M., Konzelmann, T., Calpini, B., & Clot, B. (2016). All-optical automatic pollen identification: Towards an operational system. Atmospheric Environment, 140, 202–212. https://doi.org/10.1016/j.atmosenv.2016.05.062

Czernik, M., Taberlet, P., Świsłocka, M., Czajkowska, M., Duda, N., & Ratkiewicz, M. (2013). Fast and efficient DNA-based method for winter diet analysis from stools of three cervids: Moose, red deer, and roe deer. Acta Theriologica, 58(4), 379–386. https://doi.org/10.1007/s13364-013-0146-9

D'Amato, G. (2000). Urban air pollution and plant-derived respiratory allergy, Clinical and Experimental Allergy, 30, 628–636. https://doi.org/10.1034/j.1398-9995.57.s72.5.x

Dalla Fior, G. (1985). La Nostra Flora, Guida alla conoscenza della flora della regione Trentino-Alto Adige, third edition, Trento: Casa Editrice G.D. Monauni

Damialis 2010. Patterns of Pollen Production and Atmospheric Circulation in the Area of Thessaloniki. PhD Thesis, School of Biology, Aristotle University of Thessaloniki

Damialis, A., Fotiou, C., Halley, J. M., & Vokou, D. (2011). Effects of environmental factors on pollen production in anemophilous woody species, Trees, 25(2), 253–264. https://doi.org/10.1007/s00468-010-0502-1

Damialis, A., Kaimakamis, E., Konoglou, M., & Akritidis, I. (2017). Estimating the abundance of airborne pollen and fungal spores at variable elevations using an aircraft : how high can they fly?. Scientific Reports, 7, 44535. https://doi.org/10.1038/srep44535

Damialis, A., Halley, J. M., Gioulekas, D., & Vokou, D. (2007). Long-term trends in atmospheric pollen levels in the city of Thessaloniki, Greece. Atmospheric Environment, 41(33), 7011–7021. https://doi.org/10.1016/j.atmosenv.2007.05.009

Darby, B. J., Todd, T. C., & Herman, M. A. (2013). High–throughput amplicon sequencing of rRNA genes requires a copy number correction to accurately reflect the effects of management practices on soil nematode community structure. Molecular ecology, 22(21), 5456-5471. https://doi.org/10.1111/mec.12480

De Barba, M., Miquel, C., Boyer, F., Mercier, C., Rioux, D., Coissac, E., et al. (2014). DNA metabarcoding multiplexing and validation of data accuracy for diet assessment: Application to omnivorous diet. Molecular Ecology Resources, 14(2), 306–323. https://doi.org/10.1111/1755-0998.12188

De La Torre, A. R., Birol, I., Bousquet, J., Ingvarsson, P. K., Jansson, S., Jones, S. J. M., et al. (2014). Insights into Conifer Giga-Genomes. Plant Physiology, 166(4), 1724–1732. https://doi.org/10.1104/pp.114.248708

De Vere, N., Jones, L. E., Gilmore, T., Moscrop, J., Lowe, A., Smith, D., et al. (2017). Using DNA metabarcoding to investigate honey bee foraging reveals limited flower use despite high floral availability. Scientific Reports, 7, 42838. https://doi.org/10.1038/srep42838

Deiner K, Bik HM, Mächler E, Seymour, M., Lacoursière- Roussel, A., Altermatt, F., et al. (2017) Environmental DNA metabarcoding: transforming how we survey animal and plant communities. Molecular Ecology 26, 5872-5895. https://doi.org/10.1111/mec.14350

Dell'Anna, R., Cristofori, A., Gottardini, E. & Monti, F. (2010). A critical presentation of innovative techniques for automated pollen identification in aerobiological monitoring networks. In B.J. Kaiser Pollen: Structure, types and effects, (pp. 273-288). New York: Nova science

Després, V. R., Nowoisky, J. F., Andreae, M. O., Klose, M., Conrad, R., & Pöschl, U. (2007). Characterization of primary biogenic aerosol particles in urban, rural, and high-alpine air by DNA sequence and restriction fragment analysis of ribosomal RNA genes. Biogeosciences, 4(6), 1127–1141. https://doi.org/10.5194/bg-4-1127-2007

Dineen, S. M., Aranda, IV, R. A., Anders, D. L., & Robertson, J. M. (2010). An evaluation of commercial DNA extraction kits for the isolation of bacterial spore DNA from soil. Journal of applied microbiology, 109(6), 1886–1896. https://doi.org/10.1111/j.1365-2672.2010.04816.x

Doolittle, W. F., & Papke, R. T. (2006). Genomics and the bacterial species problem. Genome Biology, 7(9), 116. http://doi.org/10.1186/gb-2006-7-9-116

Durham, O. C. (1946). The volumetric incidence of atmospheric allergens: IV. A proposed standard method of gravity sampling, counting, and volumetric interpolation of results. Journal of Allergy, 17(2), 79-86. https://doi.org/10.1016/0021-8707(46)90025-1

Edgar, R.C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research, 32(5), 1792–1797. http://doi.org/10.1093/nar/gkh340

Edgar, R. C. (2013). UPARSE: Highly accurate OTU sequences from microbial amplicon reads. Nature Methods, 10(10), 996–998. https://doi.org/10.1038/nmeth.2604

Elshahed, M. S., Youssef, N. H., Spain, A. M., Sheik, C., Najar, F. Z., Sukharnikov, L. O., et al. (2008). Novelty and uniqueness patterns of rare members of the soil biosphere. Applied and Environmental Microbiology, 74(17), 5422–5428. https://doi.org/10.1128/AEM.00410-08

Engelbrektson, A., Kunin, V., Wrighton, K. C., Zvenigorodsky, N., Chen, F., Ochman, H., & Hugenholtz, P. (2010). Experimental factors affecting PCR-based estimates of microbial species richness and evenness. ISME Journal, 4(5), 642–647. https://doi.org/10.1038/ismej.2009.153

Epp, L. S., Boessenkool, S., Bellemain, E. P., Haile, J., Esposito, A., Riaz, T., et al. (2012). New environmental metabarcodes for analysing soil DNA: Potential for studying past and present ecosystems. Molecular Ecology, 21(8), 1821–1833. https://doi.org/10.1111/j.1365-294X.2012.05537.x

Evans, N. T., Olds, B. P., Renshaw, M. A., Turner, C. R., Li, Y., Jerde, C. L., et al. (2016). Quantification of mesocosm fish and amphibian species diversity via environmental DNA metabarcoding. Molecular ecology resources, 16(1), 29-41. https://doi.org/10.1111/1755-0998.12433

Fadrosh, D. W., Ma, B., Gajer, P., Sengamalay, N., Ott, S., Brotman, R. M., et al. (2014). An improved dual-indexing approach for multiplexed 16S rRNA gene sequencing on the Illumina MiSeq platform. Microbiome, 2(1), 6. https://doi.org/10.1186/2049-2618-2-6

Faegri, K., & Iversen, J. (1989). Textbook of pollen analysis. London: Wiley

Festi F. & Prosser F. (2000) La flora del Parco Naturale Paneveggio Pale di San Martino. Atlante corologico e repertorio delle segnalazioni, Trento.

Ficetola, G. F., Pansu, J., Bonin, A., Coissac, E., Giguet-Covex, C., De Barba, M., et al. (2015). Replication levels, false presences and the estimation of the presence/absence from eDNA metabarcoding data. Molecular Ecology Resources, 15(3), 543–556. https://doi.org/10.1111/1755-0998.12338

Fliegerova, K., Tapio, I., Bonin, A., Mrazek, J., Callegari, M. L., Bani, P., et al. (2014). Effect of DNA extraction and sample preservation method on rumen bacterial population. Anaerobe, 29, 80–84. https://doi.org/10.1016/j.anaerobe.2013.09.015

Flokas A. 1994. Lessons of Meteorology and Climatology, Thessaloniki: Ziti (in greek)

Folloni, S., Kagkli, D. M., Rajcevic, B., Guimaraes, N. C. C., Van Droogenbroeck, B., Valicente, F. H., et al. (2012). Detection of airborne genetically modified maize pollen by real-time PCR. Molecular Ecology Resources, 12(5), 810–821. https://doi.org/10.1111/j.1755-0998.2012.03168.x

Fonseca, V. G., Nichols, B., Lallias, D., Quince, C., Carvalho, G. R., Power, D. M., et al. (2012). Sample richness and genetic diversity as drivers of chimera formation in nSSU metagenetic analyzes. Nucleic Acids Research, 40(9), e66-e66. https://doi.org/10.1093/nar/gks002

Fornaciari, M., Galan, C., Mediavilla, A., Dominquez, E., & Romano, B. (2000). Aeropalynological and phenological study in two different Mediterranean olive areas: Cordoba (Spain) and Perugia (Italy). Plant Biosystems, 134(2), 199–204. https://doi.org/10.1080/11263500012331358474

Frankland A.W. 1991. Aerobiology in Medicine. Grana 30(1), 19-23. https://doi.org/10.1080/00173139109427764

Frenguelli, G., Tedeschini, E., Veronesi, F., & Bricchi, E. (2002). Airborne pine (Pinus spp.) pollen in the atmosphere of Perugia (Central Italy): Behaviour of pollination in the two last decades. Aerobiologia, 18(3–4), 223–228. https://doi.org/10.1023/A:1021320128458

Frenz, D. A. (2000). The effect of windspeed on pollen and spore counts collected with the Rotorod Sampler and Burkard spore trap. Annals of Allergy, Asthma and Immunology, 85(5), 392–394. https://doi.org/10.1016/S1081-1206(10)62553-7

Galán, C., Smith, M., Thibaudon, M., Frenguelli, G., Oteros, J., Gehrig, R., … Brandao, R. (2014). Pollen monitoring: minimum requirements and reproducibility of analysis. Aerobiologia, 30(4), 385–395. https://doi.org/10.1007/s10453-014-9335-5

Galimberti, A., De Mattia, F., Bruni, I., Scaccabarozzi, D., Sandionigi, A., Barbuto, M., et al. (2014). A DNA barcoding approach to characterize pollen collected by honeybees. PLoS ONE, 9(10), e109363. https://doi.org/10.1371/journal.pone.0109363

Gaspar, J. M., & Thomas, W. K. (2013). Assessing the Consequences of Denoising Marker-Based Metagenomic Data. PLoS ONE, 8(3), e60458. https://doi.org/10.1371/journal.pone.0060458

Geml, J., Laursen, G. A., Timling, I., McFarland, J. M., Booth, M. G., Lennon, N., et al. (2009). Molecular phylogenetic biodiversity assessment of arctic and boreal ectomycorrhizal Lactarius Pers. (Russulales; Basidiomycota) in Alaska, based on soil and sporocarp DNA. Molecular Ecology, 18(10), 2213–2227. https://doi.org/10.1111/j.1365-294X.2009.04192.x

Georgolopoulos, G., Parducci, L., & Drouzas, A. D. (2016). A short phylogenetically informative cpDNA fragment for the identification of Pinus species. Biochemical Systematics and Ecology, 66, 166–172. https://doi.org/10.1016/j.bse.2016.03.001

Ghitarrini, S., Galán, C., Frenguelli, G. & Tedeschini, E. (2017) Phenological analysis of grasses (Poaceae) as a support for the dissection of their pollen season in Perugia (Central Italy) Aerobiologia 33(3), 339. https://doi.org/10.1007/s10453-017-9473-7

Ghitarrini, S., Pierboni, E., Rondini, C., Tedeschini, E., Tovo, G. R., Frenguelli, G., et al. (2018). New biomolecular tools for aerobiological monitoring: Identification of major allergenic Poaceae species through fast real-time PCR. Ecology and Evolution, *8*(8), 3996-4010. https://doi.org/10.1002/ece3.3891

Gibson, J. F., Shokralla, S., Curry, C., Baird, D. J., Monk, W. A., King, I., & Hajibabaei, M. (2015). Large-scale biomonitoring of remote and threatened ecosystems via high-throughput sequencing. PLoS ONE, 10(10), 1–15. https://doi.org/10.1371/journal.pone.0138432

Giovanetti, M., & Aronne, G. (2011). Honey bee interest in flowers with anemophilous characteristics: First notes on handling time and routine on Fraxinus ornus and Castanea sativa. Bulletin of Insectology, 64(1), 77–82.

González Parrado, Z., Fernández-González, D., Camazón, B., Valencia-Barrera, R. M., Vega-Maray, A. M., Asturias, J. a., Monsalve R. I. & Mandrioli, P. (2014). Molecular aerobiology – Plantago allergen Pla l 1 in the atmosphere. Annals of Agricultural and Environmental Medicine, 21(2), 282–289. https://doi.org/10.5604/1232-1966.1108592

Gottardini, E., Rossi, S., Cristofolini, F., & Benedetti, L. (2007). Use of Fourier transform infrared (FT-IR) spectroscopy as a tool for pollen identification. Aerobiologia, 23(3), 211–219. https://doi.org/10.1007/s10453-007-9065-z

Gottardini, E., Cristofolini, F., Bucher, E., Nardelli, R., Prandini, M. (1997). Pollini e Allergia in Trentino-Alto Adige. A.P.P.A. di Bolzano, A.P.P.A. di Trento and Agenzia provinciale per la protezione dell'ambiente e la tutela del lavoro.

Guertler, P., Eicheldinger, A., Muschler, P., Goerlich, O., & Busch, U. (2014). Automated DNA extraction from pollen in honey. Food Chemistry, 149, 302–306. https://doi.org/10.1016/j.foodchem.2013.10.129

Haas, B. J., Gevers, D., Earl, A. M., Feldgarden, M., Ward, D. V., Giannoukos, G., et al. (2011). Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. Genome Research, 21(3), 494–504. https://doi.org/10.1101/gr.112730.110

Haselhorst, D. S., Moreno, J. E., & Punyasena, S. W. (2013). Variability within the 10-year pollen rain of a seasonal neotropical forest and its implications for paleoenvironmental and phenological research. PloS One, 8(1), e53485. https://doi.org/10.1371/journal.pone.0053485

Hawkins, J., De Vere, N., Griffith, A., Ford, C. R., Allainguillaume, J., Hegarty, M. J., et al. (2015). Using DNA metabarcoding to identify the floral composition of honey: A new tool for investigating honey bee foraging preferences. PLoS ONE, 10(8), 1–20. https://doi.org/10.1371/journal.pone.0134735

Head, S., Komori, K., LaMere, S., Whisenant, T., Van Nieuwerburgh, F., Salomon, D. R., et al. (2014). Library construction for next-generation sequencing: Overviews and challenges. Biotechniques, 56(2), 61-77. https://doi.org/10.2144/000114133.Library

Hebert, P. D. N., Cywinska, A., Ball, S. L., & deWaard, J. R. (2003). Biological identifications through DNA barcodes. Proceedings of the Royal Society B: Biological Sciences, 270(1512), 313–321. https://doi.org/10.1098/rspb.2002.2218

Henderson, G., Cox, F., Kittelmann, S., Miri, V. H., Zethof, M., Noel, S. J., et al. (2013). Effect of DNA extraction methods and sampling techniques on the apparent structure of cow and sheep rumen microbial communities. PloS One, 8(9), e74787. https://doi.org/10.1371/journal.pone.0074787

Hicks, S. (2001). The use of annual arboreal pollen deposition values for delimiting tree-lines in the landscape and exploring models of pollen dispersal. Review of Palaeobotany and Palynology, 117(1–3), 1–29. https://doi.org/10.1016/S0034-6667(01)00074-4

Hicks, S. (2006). When no pollen does not mean no trees. Vegetation History and Archaeobotany, 15(4), 253–261. https://doi.org/10.1007/s00334-006-0063-9

Hicks, S., Tinsley, H., Huusko, A., Jensen, C., Hattestrand, M., Gerasimides, A., et al. (2001). Some comments on spatial variation in arboreal pollen deposition: First records from the Pollen Monitoring Programme (PMP). Review of Palaeobotany and Palynology, 117(1–3), 183–194. https://doi.org/10.1016/S0034-6667(01)00086-0

Hiiesalu, I., Öpik, M., Metsis, M., Lilje, L., Davison, J., Vasar, M., et al. (2012). Plant species richness belowground: Higher richness and new patterns revealed by next-generation sequencing. Molecular Ecology, 21(8), 2004–2016. https://doi.org/10.1111/j.1365-294X.2011.05390.x

Hirst, J. M. (1952). An automated volumetric spore trap. Annals of Applied Biology, 39(2), 257–265. https://doi.org/10.1111/j.1744-7348.1952.tb00904.x

Hofmann, F., Epp, R., Kalchschmid, A., Kratz, W., Kruse, L., Kuhn, U., et al. (2010). Monitoring of Bt-Maize pollen exposure in the vicinity of the nature reserve Ruhlsdorfer Bruch in northeast Germany 2007 to 2008. Umweltwissenschaften Und Schadstoff-Forschung, 22(3), 229–251. https://doi.org/10.1007/s12302-010-0133-6

Hollingsworth, P. M., Li, D.-Z., van der Bank, M., & Twyford, A. D. (2016). Telling plant species apart with DNA: from barcodes to genomes. Philosophical Transactions of the Royal Society B: Biological Sciences, 371(1702), 20150338. https://doi.org/10.1098/rstb.2015.0338

Huber, J. A., Morrison, H. G., Huse, S. M., Neal, P. R., Sogin, M. L., & Mark Welch, D. B. (2009). Effect of PCR amplicon size on assessments of clone library microbial diversity and community structure. Environmental Microbiology, 11(5), 1292–1302. https://doi.org/10.1111/j.1462-2920.2008.01857.x

Huse, S. M., Welch, D. M., Morrison, H. G., & Sogin, M. L. (2010). Ironing out the wrinkles in the rare biosphere through improved OTU clustering. Environmental Microbiology, 12(7), 1889–1898. https://doi.org/10.1111/j.1462-2920.2010.02193.x

Hwang, K., Oh, J., Kim, T. K., Kim, B. K., Yu, D. S., Hou, B. K., et al. (2013). CLUSTOM: A Novel Method for Clustering 16S rRNA Next Generation Sequences by Overlap Minimization. PLoS ONE, 8(5). https://doi.org/10.1371/journal.pone.0062623

Ihrmark, K., Bödeker, I. T. M., Cruz-Martinez, K., Friberg, H., Kubartova, A., Schenck, J., et al. (2012). New primers to amplify the fungal ITS2 region - evaluation by 454-sequencing of artificial and natural communities. FEMS Microbiology Ecology, 82(3), 666–677. https://doi.org/10.1111/j.1574-6941.2012.01437.x

Jato, V., Méndez, J., Rodríguez-Rajo, J., & Seijo, C. (2002). The relationship between the flowering phenophase and airborne pollen of Betula in galicia (N.W. Spain). Aerobiologia, 18(1), 55–64. https://doi.org/10.1023/A:1014987325946

Jochner, S., Ziello, C., Böck, A., Estrella, N., Buters, J., Weichenmeier, I., et al. (2012). Spatio-temporal investigation of flowering dates and pollen counts in the topographically complex Zugspitze area on the German-Austrian border. Aerobiologia, 28(4), 541–556. https://doi.org/10.1007/s10453-012-9255-1

Jones, A. M., & Harrison, R. M. (2004). The effects of meteorological factors on atmospheric bioaerosol concentrations—a review. Science of the Total Environment, 326(1-3), 151-180. https://doi.org/10.1016/j.scitotenv.2003.11.021

Jørgensen, T., Haile, J., & Moller, P. (2012) A comparative study of ancient sedimentary DNA, pollen and macrofossils from permafrost sediments of northern Siberia reveals long-term vegetational stability, Molecular Ecology, 21(8), 1989–2003. https://doi.org/10.1111/j.1365-294X.2011.05287.x

Kchouk, M., Gibrat, J. F., & Elloumi, M. (2017). Generations of Sequencing Technologies: From First to Next Generation. Biology and Medicine, 9(3), 1000395. https://doi.org/10.4172/0974-8369.1000395

Keller, A., Danner, N., Grimmer, G., Ankenbrand, M., von der Ohe, K., von der Ohe, W., et al. (2015). Evaluating multiplexed next-generation sequencing as a method in palynology for mixed pollen samples. Plant Biology, 17(2), 558–566. https://doi.org/10.1111/plb.12251

Kembel, S. W., Wu, M., Eisen, J. A., & Green, J. L. (2012). Incorporating 16S Gene Copy Number Information Improves Estimates of Microbial Diversity and Abundance. PLoS Computational Biology, 8(10), 16–18. https://doi.org/10.1371/journal.pcbi.1002743

Kennedy, N. A., Walker, A. W., Berry, S. H., Duncan, S. H., Farquarson, F. M., Louis, P., et al. (2014). The Impact of Different DNA Extraction Kits and Laboratories upon the Assessment of Human Gut Microbiota Composition by 16S rRNA Gene Sequencing. PLoS ONE, 9(2), e88982. https://doi.org/10.1371/journal.pone.0088982

Khanduri, V. P., & Sharma, C. M. (2000). Development of groups of male strobili, anthesis and microsporangium dehiscence in Pinus roxburghii. Grana, 39(4), 169–174. https://doi.org/10.1080/00173130051084269

Koljalg, U., Nilsson, R. H., Abarenkov, K., Tedersoo, L., Taylor, A. F. S., & Bahram, M. (2014). Towards a unified paradigm for sequence-based identification of fungi. Molecular Ecology, 22(21), 5271–5277. https://doi.org/10.1111/mec.12481

Kowalczyk, R., Taberlet, P., Coissac, E., Valentini, A., Miquel, C., Kamiński, T., et al. (2011). Influence of management practices on large herbivore diet-Case of European bison in Białowieza Primeval Forest (Poland). Forest Ecology and Management, 261(4), 821–828. https://doi.org/10.1016/j.foreco.2010.11.026

Kraaijeveld, K., de Weger, L. A., García, M. V., Buermans, H., Frank, J., Hiemstra, P. S., et al. (2015). Efficient and sensitive identification and quantification of airborne pollen using next-generation DNA sequencing. Molecular Ecology Resources, 15(1), 8–16. https://doi.org/10.1111/1755-0998.12288

Kunin, V., Engelbrektson, A., Ochman, H., & Hugenholtz, P. (2010). Wrinkles in the rare biosphere: Pyrosequencing errors can lead to artificial inflation of diversity estimates. Environmental Microbiology, 12(1), 118–123. https://doi.org/10.1111/j.1462-2920.2009.02051.x

Kvavadze, E. (2001). Annual modern pollen deposition in the foothills of the Lagodekhi Reservation (Caucasus, East Georgia), 41(2), 355–364. https://doi.org/10.1080/00173137909428871

Lacey, M. & West, J. (2006). The air spora. A manual for catching and identifying airborne biological particles, Springer, Dordrecht.

Laha, R. C., De Mandal, S., Ralte, L., Ralte, L., Kumar, N. S., Gurusubramanian, G., et al. (2017). Meta-barcoding in combination with palynological inference is a potent diagnostic marker for honey floral composition. AMB Express, 7(1), 132. https://doi.org/10.1186/s13568-017-0429-7

Lallias, D., Hiddink, J. G., Fonseca, V. G., Gaspar, J. M., Sung, W., Neill, S. P., et al. (2015). Environmental metabarcoding reveals heterogeneous drivers of microbial eukaryote diversity in contrasting estuarine ecosystems. ISME Journal, 9(5), 1208–1221. https://doi.org/10.1038/ismej.2014.213

Lazarevic, V., Gaïa, N., Girard, M., François, P., & Schrenzel, J. (2013). Comparison of DNA Extraction Methods in Analysis of Salivary Bacterial Communities. PLoS ONE, 8(7), e67699. https://doi.org/10.1371/journal.pone.0067699

Lekberg, Y., Gibbons, S. M., & Rosendahl, S. (2014). Will different OTU delineation methods change interpretation of arbuscular mycorrhizal fungal community patterns? New Phytologist, 202(4), 1101–1104. https://doi.org/10.1111/nph.12758

Leontidou, K., Vernesi, C., De Groeve, J., Cristofolini, F., Vokou, D. & Cristofori, A. (2018). DNA metabarcoding of airborne pollen: new protocols for improved taxonomic identification of environmental samples. Aerobiologia, 34(1), 63-74. https://doi.org/10.1007/s10453-017-9497-z

Leray, M., & Knowlton, N. (2015). DNA barcoding and metabarcoding of standardized samples reveal patterns of marine benthic diversity. Proceedings of the National Academy of Sciences, 112(7), 2076–2081. https://doi.org/10.1073/pnas.1424997112

Levetin, E., Rogers, C. A., & Hall, S. A. (2000). Comparison of pollen sampling with a Burkard spore trap and a Tauber trap in a warm temperate climate. Grana, 39(6), 294–302. https://doi.org/10.1080/00173130052504333

Levetin, E. (2004). Methods for aeroallergen sampling. Current Allergy and Asthma Reports, 4(5), 376–83. https://doi.org/10.1007/s11882-004-0088-z

Lewis, W. H., Vinay, P., & Zenger, V. E. (1983). Airborne and allergenic pollen of North America. USA: The Johns Hopkins University Press.

Lim, N. K. M., Tay, Y. C., Srivathsan, A., Tan, J. W. T., Kwik, J. T. B., Baloğlu, B., et al. (2016). Next-generation freshwater bioassessment: eDNA metabarcoding with a conserved metazoan primer reveals species-rich and reservoir-specific communities. Royal Society Open Science, 3(11), 160635. https://doi.org/10.1098/rsos.160635

Longhi, S., Cristofori, A., Gatto, P., Cristofolini, F., Grando, M. S., & Gottardini, E. (2009). Biomolecular identification of allergenic pollen: A new perspective for aerobiological monitoring? Annals of Allergy, Asthma and Immunology, 103(6), 508–514. https://doi.org/10.1016/S1081-1206(10)60268-2

Luo, C., Tsementzi, D., Kyrpides, N., Read, T., & Konstantinidis, K. T. (2012). Direct comparisons of Illumina vs. Roche 454 sequencing technologies on the same microbial community DNA sample. PLoS ONE, 7(2), e30087. https://doi.org/10.1371/journal.pone.0030087

Mandrioli, P. (1998). Basic aerobiology. Aerobiologia, 14(2), 89–94. https://doi.org/10.1007/bf02694191

Mardis, E. R. (2008). The impact of next-generation sequencing technology on genetics. Trends in Genetics, 24(3), 133–141. https://doi.org/10.1016/j.tig.2007.12.007

Mardis, E. R. (2013). Next-Generation Sequencing Platforms. Annual Review of Analytical Chemistry, 6(1), 287–303. https://doi.org/10.1146/annurev-anchem-062012-092628

McFrederick, Q. S., & Rehan, S. M. (2016). Characterization of pollen and bacterial community composition in brood provisions of a small carpenter bee. Molecular Ecology, 25(10), 2302–2311. https://doi.org/10.1111/mec.13608

Menzel, A., & Fabian, P. (1999). Growing season extended in Europe. Nature, 397(6721), 659–659. https://doi.org/10.1038/17709

Menzel, A. (2000). Trends in phenological phases in Europe between 1951 and 1996. International Journal of Biometeorology, 44(2), 76–81. https://doi.org/10.1007/s004840000054

Menzel, A., Sparks, T. H., Estrella, N., & Roy, D. B. (2006). Altered geographic and temporal variability in phenology in response to climate change. Global Ecology and Biogeography, 15(5), 498–504. https://doi.org/10.1111/j.1466-822X.2006.00247.x

Mohanty, RP, Buchheim, MA, Anderson, J & Levetin, E (2017) Molecular analysis confirms the long-distance transport of Juniperus ashei pollen. PLoS ONE 12(3), e0173465. https://doi.org/10.1371/journal.pone.0173465

Moore PD, Webb JA, Collinson ME (1991) Pollen Analysis. 2nd ed. Blackwell Scientific Publications, London.

Morgan, M. J., Chariton, A. A., Hartley, D. M., Court, L. N., & Hardy, C. M. (2013). Improved Inference of Taxonomic Richness from Environmental DNA. PLoS ONE, 8(8). https://doi.org/10.1371/journal.pone.0071974

Müller, F., & Rieu, I. (2016). Acclimation to high temperature during pollen development. Plant Reproduction, 29(1–2), 107–118. https://doi.org/10.1007/s00497-016-0282-x

Murray, D. C., Coghlan, M. L., & Bunce, M. (2015). From benchtop to desktop: Important considerations when designing amplicon sequencing workflows. PLoS ONE, 10(4), 1–21. https://doi.org/10.1371/journal.pone.0124671

Nilsson, R. H., Kristiansson, E., Ryberg, M., & Hallenberg, N. (2008). Intraspecific ITS Variability in the Kingdom Fungi as Expressed in the International Sequence Databases and Its Implications for Molecular Species Identification, Evolutionary Bioinformatics, 4, 193–201. https://doi.org/10.4137/EBO.S653

Núñez, A., de Paz, G. A., Ferencova, Z., Rastrojo, A., Guantes, R., García, A. M., et al. (2017). Validation of the Hirst-type spore trap for simultaneous monitoring of prokaryotic and eukaryotic biodiversities in urban air samples by next-generation sequencing. Applied and Environmental Microbiology, 83(13), e00472-17. https://doi.org/10.1128/AEM.00472-17

Ogden, E. C., & Raynor, G. S. (1967). A new sampler for airborne pollen: the rotoslide. Journal of Allergy, 40(1), 1-11. https://doi.org/10.1016/0021-8707(67)90053-6

Oksanen, J., Blanchet F. G., Friendly M., Kindt, R., Legendre, P., Dan McGlinn, et al. (2017). vegan: Community Ecology Package. R package Version 2.4–3. http://CRAN. R-project. org/package= vegan.

Olds, B. P., Jerde, C. L., Renshaw, M. A., Li, Y., Evans, N. T., Turner, C. R., et al. (2016). Estimating species richness using environmental DNA. Ecology and Evolution, 6(12), 4214–4226. https://doi.org/10.1002/ece3.2186

Ollerton, J., Winfree, R., & Tarrant, S. (2011). How many flowering plants are pollinated by animals? Oikos, 120(3), 321–326. https://doi.org/10.1111/j.1600-0706.2010.18644.x

Olsgard, F., Somerfield, P. J., & Carr, M. R. (1998). Relationships between taxonomic resolution, macrobenthic community patterns and disturbance. Marine Ecology Progress Series, 172, 25–36. https://doi.org/10.3354/meps172025

Osborne, C. P., Chuine, I., Viner, D., & Woodward, F. I. (2000). Olive phenology as a sensitive indicator of future climatic warming in the Mediterranean. Plant, Cell and Environment, 23(7), 701–710. https://doi.org/10.1046/j.1365-3040.2000.00584.x

Oteros, J., García-Mozo, H., Alcázar, P., Belmonte, J., Bermejo, D., Boi, M.,et al. (2015). A new method for determining the sources of airborne particles. Journal of Environmental Management, 155, 212–218. https://doi.org/10.1016/j.jenvman.2015.03.037

Pappas, C. S., Tarantilis, P. A., Harizanis, P. C., & Polissiou, M. G. (2003). New Method for Pollen Identication by FT-IR Spectroscopy. Applied Spectroscopy, 57(1), 23–27. https://doi.org/10.1366/000370203321165160

Parducci, L., Bennett, K. D., Ficetola, G. F., Alsos, I. G., Suyama, Y., Wood, J. R., et al. (2017). Ancient plant DNA in lake sediments. New Phytologist, 214(3), 924–942. https://doi.org/10.1111/nph.14470

Parducci, L., Matetovici, I., Fontana, S. L., Bennett, K. D., Suyama, Y., Haile, J., et al. (2013). Molecular- and pollen-based vegetation analysis in lake sediments from central Scandinavia. Molecular Ecology, 22(13), 3511–3524. https://doi.org/10.1111/mec.12298

Parducci, L., Suyama, Y., Lascoux, M., & Bennett, K. D. (2005). Ancient DNA from pollen: A genetic record of population history in Scots pine. Molecular Ecology, 14(9), 2873–2882. https://doi.org/10.1111/j.1365-294X.2005.02644.x

Patin, N. V., Kunin, V., Lidström, U., & Ashby, M. N. (2013). Effects of OTU Clustering and PCR Artifacts on Microbial Diversity Estimates. Microbial Ecology, 65(3), 709–719. https://doi.org/10.1007/s00248-012-0145-4

Peccia, J., & Hernandez, M. (2006). Incorporating polymerase chain reaction-based identification, population characterization, and quantification of microorganisms into aerosol science: A review. Atmospheric Environment, 40(21), 3941–3961. https://doi.org/10.1016/j.atmosenv.2006.02.029

Pedersen B. V., Moseholm L. 1993. Precision of the daily pollen count. Identifying sources of variation using variance component models. Aerobiologia, 9(1), 15-26. https://doi.org/10.1007/BF02311366

Pegard, A., Miquel, C., Valentini, A., Coissac, E., Bouvier, F., François, D., et al. (2009). Universal DNA-based methods for assessing the diet of grazing livestock and wildlife from feces. Journal of Agricultural and Food Chemistry, 57(13), 5700–5706. https://doi.org/10.1021/jf803680c

Piano del Parco, Parco Naturale Paneveggio Pale di San Martino, Relazione Illustrativa, Provincia Autonoma di Trento, 2012

Pidek I.A., Piotrowska, K. & Kasprzyk, I. (2010). Pollen–vegetation relationships for pine and spruce in southeast Poland on the basis of volumetric and Tauber trap records. Grana, 49(3), 215-226 https://doi.org/10.1080/00173134.2010.514006

Pöhlker, C., Huffman, J. A., & Pöschl, U. (2013). Autofluorescence of atmospheric bioaerosols: Spectral fingerprints and taxonomic trends of pollen. Atmospheric Measurement Techniques, 6(12), 3369–3392. https://doi.org/10.5194/amt-6-3369-2013

Pompanon, F., Deagle, B. E., Symondson, W. O. C., Brown, D. S., Jarman, S. N., & Taberlet, P. (2012). Who is eating what: Diet assessment using next generation sequencing. Molecular Ecology, 21(8), 1931–1950. https://doi.org/10.1111/j.1365-294X.2011.05403.x

Pornon, A., Escaravage, N., Burrus, M., Holota, H., Khimoun, A., Mariette, J., et al. (2016). Using metabarcoding to reveal and quantify plant-pollinator interactions. Scientific Reports, 6, 27282. https://doi.org/10.1038/srep27282

Portnoy, J., Landuyt, J., Pacheco, F., Flappan, S., Simon, S., & Barnes, C. (2000). Comparison of the Burkard and Allergenco MK-3 volumetric collectors. Annals of allergy, Asthma & Immunology, 84(1), 19-24. https://doi.org/10.1016/S1081-1206(10)62735-4

Preheim, S. P., Perrott, A. R., Martin-Platero, A. M., Gupta, A., & Alm, E. J. (2013). Distribution-based clustering: Using ecology to refine the operational taxonomic unit. Applied and Environmental Microbiology, 79(21), 6593–6603. https://doi.org/10.1128/AEM.00342-13

Primavesi, L. F., Wu, H., Mudd, E. A., Day, A., & Jones, H. D. (2017). Visualisation of plastid degradation in sperm cells of wheat pollen. Protoplasma, 254(1), 229–237. https://doi.org/10.1007/s00709-015-0935-x

Prosser, J. I. (2010). Replicate or lie. Environmental Microbiology, 12(7), 1806–1810. https://doi.org/10.1111/j.1462-2920.2010.02201.x

Schmidt-Kloiber A. & Nijboer R.C. (2004) The Effect of Taxonomic Resolution on the Assessment of Ecological Water Quality Classes. Hydrobiologia, 516(1-3), 269-283. https://doi.org/10.1023/B:HYDR.0000025270.10807.10

Quail, M. A., Smith, M., Coupland, P., Otto, T. D., Harris, S. R., Connor, T. R., et al. (2012). A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers. BMC Genomics, 13(1), 1. https://doi.org/10.1186/1471-2164-13-341

Quince, C., Lanzén, A., Curtis, T. P., Davenport, R. J., Hall, N., Head, I. M., et al. (2009). Accurate determination of microbial diversity from 454 pyrosequencing data. Nature Methods, 6(9), 639–641. https://doi.org/10.1038/nmeth.1361

R Core Team (2016) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Rathcke, B., & Lacey, E. P. (1985). Phenological Patterns of Terrestrial Plants. Annual Review of Ecology and Systematics, 16(1), 179–214. https://doi.org/10.1146/annurev.es.16.110185.001143

Rayé, G., Miquel, C., Coissac, E., Redjadj, C., Loison, A., & Taberlet, P. (2011). New insights on diet variability revealed by DNA barcoding and high-throughput pyrosequencing: chamois diet in autumn as a case study. Ecological Research, 26(2), 265-276. https://doi.org/10.1007/s11284-010-0780-5

Reid, C. E., & Gamble, J. L. (2009). Aeroallergens, allergic disease, and climate change: Impacts and adaptation. EcoHealth, 6(3), 458–470. https://doi.org/10.1007/s10393-009-0261-x

Richardson, B. A., Chaney, L., Shaw, N. L., & Still, S. M. (2017). Will phenotypic plasticity affecting flowering phenology keep pace with climate change? Global Change Biology, 23(6), 2499–2508. https://doi.org/10.1111/gcb.13532

Richardson, R. T., Lin, C.-H., Sponsler, D. B., Quijia, J. O., Goodell, K., & Johnson, R. M. (2015). Application of ITS2 Metabarcoding to Determine the Provenance of Pollen Collected by Honey Bees in an Agroecosystem. Applications in Plant Sciences, 3(1), 1400066. https://doi.org/10.3732/apps.1400066

Richardson, R. T., Lin, C.-H., Quijia, J. O., Riusech, N. S., Goodell, K., & Johnson, R. M. (2015b). Rank-Based Characterization of Pollen Assemblages Collected by Honey Bees Using a Multi-Locus Metabarcoding Approach. Applications in Plant Sciences, 3(11), 1500043. https://doi.org/10.3732/apps.1500043

Rodríguez-Rajo, F. J., Aira, M. J., Fernández-González, M., Seijo, C., & Jato, V. (2011). Recent trends in airborne pollen for tree species in Galicia, NW Spain. Climate Research, 48(2–3), 281–291. https://doi.org/10.3354/cr00966

Rognes T, Flouri T, Nichols B, Quince, C., Mahè, F. (2016) VSEARCH: a versatile open source tool for metagenomics. PeerJ, 4, e2584. https://doi.org/10.7717/peerj.2584

Rosen, M. J., Callahan, B. J., Fisher, D. S., & Holmes, S. P. (2012). Denoising PCR-amplified metagenome data. BMC Bioinformatics, 13(1). https://doi.org/10.1186/1471-2105-13-283

Sandionigi A, Galimberti A, Labra M, Ferri, E., Panunzi, E., De Mattia, F., et al. (2012) Analytical approaches for DNA barcoding data – how to find a way for plants? Plant Biosystems, 146(4), 805–813 http://dx.doi.org/10.1080/11263504.2012.740084

Sangwan, R. S., & Sangwan-Norreel, B. S. (1987). Ultrastructural cytology of plastids in pollen grains of certain androgenic and nonandrogenic plants. Protoplasma, 138(1), 11–22. https://doi.org/10.1007/BF01281180

Schnell, I. B., Thomsen, P. F., Wilkinson, N., Rasmussen, M., Jensen, L. R. D., Willerslev, E., et al. (2012). Erratum: Screening mammal biodiversity using dna from leeches (Current Biology (2012) 22 (R262-R263)). Current Biology, 22(20), 1980. https://doi.org/10.1016/j.cub.2012.10.014

Shehzad, W., Riaz, T., Nawaz, M. A., Miquel, C., Poillot, C., Shah, S. A., et al. (2012). Carnivore diet analysis based on next-generation sequencing: Application to the leopard cat (Prionailurus bengalensis) in Pakistan. Molecular Ecology, 21(8), 1951–1965. https://doi.org/10.1111/j.1365-294X.2011.05424.x

Shokralla S, Spall JL, Gibson JF & Hajibabaei M (2012). Next-generation sequencing technologies for environmental DNA research. Molecular Ecology, 21(8), 1794-805. https://doi.org/10.1111/j.1365-294X.2012.05538.x

Sickel W, Ankenbrand MJ, Grimmer G, Holzschuh A., Härtel, S., Lanzen, J., et al. (2015) Increased efficiency in identifying mixed pollen samples by meta-barcoding with a dual-indexing approach. BMC Ecology, 15(1), 20 https://doi.org/10.1186/s12898-015-0051-y

Sipos, R., Székely, A. J., Palatinszky, M., Révész, S., Márialigeti, K., & Nikolausz, M. (2007). Effect of primer mismatch, annealing temperature and PCR cycle number on 16S rRNA gene-targetting bacterial community analysis. FEMS Microbiology Ecology, 60(2), 341–350. https://doi.org/10.1111/j.1574-6941.2007.00283.x

Smart, M. D., Cornman, R. S., Iwanowicz, D. D., McDermott-Kubeczko, M., Pettis, J. S., Spivak, M. S., & Otto, C. R. V. (2017). A comparison of honey bee-collected pollen from working agricultural lands using light microscopy and its metabarcoding. Environmental Entomology, 46(1), 38–49. https://doi.org/10.1093/ee/nvw159

Smith, M., Jäger, S., Berger, U., Šikoparija, B., Hallsdottir, M., Sauliene, I., et al. (2014). Geographic and temporal variations in pollen exposure across Europe. Allergy: European Journal of Allergy and Clinical Immunology, 69(7), 913–923. https://doi.org/10.1111/all.12419

Sogin, M. L., Morrison, H. G., Huber, J. A., Welch, D. M., Huse, S. M., Neal, P. R., et al. (2006). Microbial diversity in the deep sea and the underexplored "rare biosphere". Proceedings of the National Academy of Sciences, 103(32), 12115-12120. https://doi.org/10.1073/pnas.0605127103

Soininen, E. M., Valentini, A., Coissac, E., Miquel, C., Gielly, L., Brochmann, C., et al. (2009). Analysing diet of small herbivores: The efficiency of DNA barcoding coupled with high-throughput pyrosequencing for deciphering the composition of complex plant mixtures. Frontiers in Zoology, 6(1), 1–9. https://doi.org/10.1186/1742-9994-6-16

Soininen, E. M., Gauthier, G., Bilodeau, F., Berteaux, D., Gielly, L., Taberlet, P., et al. (2015). Highly overlapping winter diet in two sympatric lemming species revealed by DNA metabarcoding. PLoS ONE, 10(1). https://doi.org/10.1371/journal.pone.0115335

Sonstebo, J. H., Gielly, L., Brysting, A. K., Elven, R., Edwards, M., Haile, J., et al. (2010). Using next-generation sequencing for molecular reconstruction of past Arctic vegetation and climate. Molecular Ecology Resources, 10(6), 1009–1018. https://doi.org/10.1111/j.1755-0998.2010.02855.x

Srivathsan, A., Sha, J. C. M., Vogler, A. P., & Meier, R. (2015). Comparing the effectiveness of metagenomics and metabarcoding for diet analysis of a leaf-feeding monkey (Pygathrix nemaeus). Molecular Ecology Resources, 15(2), 250–261. https://doi.org/10.1111/1755-0998.12302

Stevens, J. L., Jackson, R. L., & Olson, J. B. (2013). Slowing PCR ramp speed reduces chimera formation from environmental samples. Journal of Microbiological Methods, 93(3), 203–205. https://doi.org/10.1016/j.mimet.2013.03.013

Stoof-Leichsenring, K. R., Epp, L. S., Trauth, M. H., & Tiedemann, R. (2012). Hidden diversity in diatoms of Kenyan Lake Naivasha: A genetic approach detects temporal variation. Molecular Ecology, 21(8), 1918–1930. https://doi.org/10.1111/j.1365-294X.2011.05412.x

Taberlet, P., Gielly, L., Pautou, G., & Bouvet, J. (1991). Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Molecular Biology, 17(5), 1105–1109. https://doi.org/10.1007/BF00037152

Taberlet, P., Prud'Homme, S. M., Campione, E., Roy, J., Miquel, C., Shehzad, W., et al. (2012). Soil sampling and isolation of extracellular DNA from large amount of starting material suitable for metabarcoding studies. Molecular Ecology, 21(8), 1816–1820. https://doi.org/10.1111/j.1365-294X.2011.05317.x

Taberlet, P., Coissac, E., Pompanon, F., Gielly, L., Miquel, C., Valentini, A., et al. (2007). Power and limitations of the chloroplast trnL (UAA) intron for plant DNA barcoding. Nucleic Acids Research, 35(3), e14-e14. https://doi.org/10.1093/nar/gkl938

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Molecular Biology and Evolution, 30(12), 2725–2729. https://doi.org/10.1093/molbev/mst197

Tauber, H. (1967). Investigations of the mode of pollen transfer in forested areas. Review of Palaeobotany and Palynology, 3(1-4), 277-286. https://doi.org/10.1016/0034-6667(67)90060-7

Tringe, S. G., Zhang, T., Liu, X., Yu, Y., Lee, W. H., Yap, J., et al. (2008). The airbone metagenome in an indoor urban environment. PLoS ONE, 3(4). https://doi.org/10.1371/journal.pone.0001862

Tutin, T. G., Burges, N. A., Chater, A. O., Edmonson, J. R., Heywood, V. H., Moore, D. M., et al. (1993). Flora Europaea, Vol. 1 (2nd ed.). Cambridge: Cambridge University Press.

Tutin, T. G., Heywood, V. H., Burges, N. A., Moore, D. M., Valentine, D. H., Walters, S. M., & Webb, D. A. (1968–1980). Flora Europaea, Vol. 2–5. Cambridge: Cambridge University Press.

Valentini, A., Miquel, C., Nawaz, M. A., Bellemain, E., Coissac, E., Pompanon, F., et al. (2009). New perspectives in diet analysis based on DNA barcoding and parallel pyrosequencing: The trnL approach. Molecular Ecology Resources, 9(1), 51–60. https://doi.org/10.1111/j.1755-0998.2008.02352.x

Valentini, A., Miquel, C., & Taberlet, P. (2010). DNA barcoding for honey biodiversity. Diversity, 2(4), 610–617. https://doi.org/10.3390/d2040610

Van De Water, P. K., Keever, T., Main, C. E., & Levetin, E. (2003). An assessment of predictive forecasting of Juniperus ashei pollen movement in the Southern Great Plains, USA. International Journal of Biometeorology, 48(2), 74–82. https://doi.org/10.1007/s00484-003-0184-0

Van der Knaap, W. O., Van Leeuven, J. F. N. & Ammann, B. (2001). Seven years of annual pollen influx at the forest limit in the Swiss Alps studied by pollen traps in relation to vegetation and climate. Review of Palaeobotany and Palynology, 117(1-3), 31–52. https://doi.org/10.1016/S0034-6667(01)00075-6

Van Dijk, E. L., Jaszczyszyn, Y., & Thermes, C. (2014). Library preparation methods for next-generation sequencing : Tone down the bias. Experimental Cell Research, 322(1), 12–20. https://doi.org/10.1016/j.yexcr.2014.01.008

Venugopal, S. (2016) Biology. New Delhi:New Saraswati House

Vlčková, K., Mrázek, J., Kopečný, J., & Petrželková, K. J. (2012). Evaluation of different storage methods to characterize the fecal bacterial communities of captive western lowland gorillas (Gorilla gorilla gorilla). Journal of Microbiological Methods, 91(1), 45–51. https://doi.org/10.1016/j.mimet.2012.07.015

Vokou 2009. General Ecology. An introduction. Thessaloniki: University Studio Press (in greek)

Volkova, O., & Severova, E. (2017). A comparison of modern pollen rain collected by Hirst type and Tauber traps in Moscow, Russia, Ecological Questions, 26, 15–17. http://dx.doi.org/10.12775/EQ.2017.010

Walther, G. R., Post, E., Convey, P., Menzel, A., Parmesan, C., Beebee, T.J.C., Fromentin J.-M., et al. (2002). Ecological responses to recent climate change. Nature, 416(6879), 389-395. https://doi.org/10.1038/416389a

Wang, G. C. Y., & Wang, Y. (1996). The frequency of chimeric molecules as a consequence of PCR co- amplification of 16S rRNA genes from different bacterial species. Applied and Environmental Microbiology, 142(12), 1107–1114. https://doi.org/10.1099/13500872-142-5-1107

Werner, J. J., Zhou, D., Caporaso, J. G., Knight, R., & Angenent, L. T. (2012). Comparison of Illumina paired-end and single-direction sequencing for microbial 16S rRNA gene amplicon surveys. ISME Journal, 6(7), 1273–1276. https://doi.org/10.1038/ismej.2011.186

West, J. S., & Kimber, R. B. E. (2015). Innovations in air sampling to detect plant pathogens. Annals of Applied Biology, 166(1), 4–17. https://doi.org/10.1111/aab.12191

Whittaker, R.H. (1972). Evolution and measurement of species diversity. Taxon, 21(2-3), 213–251. doi:10.2307/1218190. https://www.jstor.org/stable/1218190

Wilson, E. E., Sidhu, C. S., Levan, K. E., & Holway, D. A. (2010). Pollen foraging behaviour of solitary Hawaiian bees revealed through molecular pollen analysis. Molecular Ecology, 19(21), 4823–4829. https://doi.org/10.1111/j.1365-294X.2010.04849.x

Winter, D. (2016). rentrez: Entrez in R. R package version 1.0.2. https://CRAN.R-project.org/package=rentrez

Wolf, A. A., Zavaleta, E. S., & Selmants, P. C. (2017). Flowering phenology shifts in response to biodiversity loss. Proceedings of the National Academy of Sciences, 114(13), 3463–3468. https://doi.org/10.1073/pnas.1608357114

Wu, J. Y., Jiang, X. T., Jiang, Y. X., Lu, S. Y., Zou, F., & Zhou, H. W. (2010). Effects of polymerase, template dilution and cycle number on PCR based 16 S rRNA diversity analysis using the deep sequencing method. BMC Microbiology, 10(1), 255. https://doi.org/10.1186/1471-2180-10-255

Xu, H., Swoboda, I., Bhalla, P. L., & Singh, M. B. (1999). Male gametic cell-specific gene expression in flowering plants. Proceedings of the National Academy of Sciences, USA, 96(5), 2554–2558. https://doi.org/VL - 96

Xu, L., Ravnskov, S., Larsen, J., & Nicolaisen, M. (2011). Influence of DNA extraction and PCR amplification on studies of soil fungal communities based on amplicon sequencing. Canadian Journal of Microbiology, 57(12), 1062–1066. https://doi.org/10.1139/w11-100

Yamamoto, S., Masuda, R., Sato, Y., Sado, T., Araki, H., Kondoh, M., et al. (2017). Environmental DNA metabarcoding reveals local fish communities in a species-rich coastal sea. Scientific Reports, 7, 40368. https://doi.org/10.1038/srep40368

Yoccoz, N. G., Bråthen, K. A., Gielly, L., Haile, J., Edwards, M. E., Goslar, T., et al. (2012). DNA from soil mirrors plant taxonomic and growth form diversity. Molecular Ecology, 21(15), 3647–3655. https://doi.org/10.1111/j.1365-294X.2012.05545.x

Yooseph, S., Andrews-Pfannkoch, C., Tenney, A., McQuaid, J., Williamson, S., Thiagarajan, M., et al. (2013). A metagenomic framework for the study of airborne microbial communities. PLoS ONE, 8(12), e81862. https://doi.org/10.1371/journal.pone.0081862

Zhou, J., Wu, L., Deng, Y., Zhi, X., Jiang, Y. H., Tu, Q., et al. (2011). Reproducibility and quantitation of amplicon sequencing-based detection. ISME Journal, 5(8), 1303–1313. https://doi.org/10.1038/ismej.2011.11

Zinger, L., Shahnavaz, B., Baptist, F., Geremia, R. A., & Choler, P. (2009). Microbial diversity in alpine tundra soils correlates with snow cover dynamics. ISME Journal, 3(7), 850–859. https://doi.org/10.1038/ismej.2009.20

Zinger, L., Gobet, A., & Pommier, T. (2012). Two decades of describing the unseen majority of aquatic microbial diversity. Molecular Ecology, 21(8), 1878–1896. https://doi.org/10.1111/j.1365-294X.2011.05362.x

Zorer, R., Eccel, E., Tognetti, R., & La Porta, N. (2014). A mediterranean conifer under vegetation shift: Seasonal changes of photochemical activity in Cupressus sempervirens (L.) and evidence of correlation with temperature models. Italian Journal of Agrometeorology, 1, 29–40. http://www.scopus.com/inward/record.url?eid=2-s2.0-84902281051&partnerID=MN8TOARS

Websites

https://www.medicinalplantsarchive.us

http://www.burkardscientific.co.uk

http://www.hund.de/en/instruments/pollen-monitor

http://www.plair.ch

http://www.454.com

http://www.illumina.com

http:// www.lifetechnologies.com

https://www.thermofisher.com/us/en/ home/brands/ion-torrent.html

https://www.OpenDataHub.it

https://www.ncbi.nlm.nih.gov/genbank

http://www.emplantbase.org/home.html

https://www. parcopan.org

http://www.comune.trento.it

https://www.paldat.org

https://www.polleninfo.org

Index of Figures

Index of Tables

Appendix

Appendix 1 Identification of pollen grains based on their morphological characteristics (https://www.polleninfo.org) for the taxa of interest in Trentino. The taxa are given grouped according to the type of aperture. The used terms are explained in the footnote (https://www.paldat.org).

Παράρτημα 1 Αναγνώριση γυρεοκόκκων με βάση μορφολογικούς χαρακτήρες (https://www.polleninfo.org) για τα τάξα ενδιαφέροντος για το Τρεντίνο. Τα τάξα δίνονται ομαδοποιημένα σύμφωνα με τον τύπο του ανοίγματος τους. Οι χρησιμοποιούμενοι όροι εξηγούνται στην υποσημείωση (https://www.paldat.org).

Taxon	No apertures	Other characteristics
Inaperturate		
Pinus	-	saccate, sacs protruding from main body, big pollen (67-87 μm)
Abies	-	saccate, sacs protruding from main body, (106-139 μm)
Picea	-	saccate, sacs enveloping the main body, (97-124 μm)
Cedrus	-	saccate, sacs enveloping the main body, (69-79 μm)
Larix	-	spheroidal, isodiametric, granular plasma with leucoplasts, (73-88 μm)
Cupressaceae-Taxaceae	-	star-shaped cytoplasma
Populus	-	spheroidal, isodiametric, exine smooth
Lauraceae	-	spheroidal, isodiametric, exine with echini
Cyperaceae	-	grain heteropolar
Porate		
Juncaceaae	1	4 grains (tetrad)
Poaceae	1	with operculum
Moraceae	2	with operculum, wide pores, wide onci, small pollen (17-20 μm)
Urticaceae	3-4	thin exine, onci, small pollen (12-13 μm)
Cannabaceae	3	thin exine, not folded, small pollen (17-21 μm)
Betula	3	thick exine, vestibulum
Ostrya	3	thick exine, spheroidal
Corylus	3	thick exine, sub-triangular
Carpinus	4-5	spheroidal
Alnus	4-5	stephanoporate, with arci
Ulmus	4-5	stephanoporate
Campanulaceae	3-7	stephanoporate, with fine echini
Pistacia	**5-7**	pantoporate, reticulate exine, poorly defined pores, fine mesh
Buxus	>16	pantoporate, reticulate exine, poorly defined pores, wide mesh
Plantaginaceae	6-10	pantoporate, verrucate exine
Juglans	11-15	pantoporate, smooth exine
Caryophyllaceae	>16	pantoporate, reticulate exine
Chenopodiaceae-Amaranthaceae	>16	pantoporate
Colpate		
Liliaceae	1	reticulate exine
Ginkgo	1	smooth exine
Ranunculaceae	3	granulate exine, with echini
Papaveraceae	3	granulate exine, regular and fine
Quercus	3	verrucate exine
Rosaceae	3	striate exine, equatorial outline sub-triangular
Acer	3	striate exine, equatorial outline sub-circular
Saxifragaceae	3	striate exine, equatorial outline sub-circular
Platanus	3	reticulate exine, furrows coarsely granular
Salix	3	reticulate exine, wide furrows, with margo
Brassicaceae	3	reticulate exine, mesh open
Fraxinus ornus	3	long furrows, reticulate exine, network reducing towards the colpi

Appendix 1 (continued)

Taxon	No apertures	Other characteristics
Colpate		
Fraxinus excelsior	3	long furrows, reticulate exine, network not reducing towards the colpi
Olea	3	prominent ornamentation
Rubiaceae	3	stephanocolpate, small pollen (15-19 µm)
Colporate		
Ericaceae	3	4 grains (tetrad)
Apiaceae	3	smooth exine (scabrate), grain prolate
Castanea	3	smooth exine (psilate), grain prolate, slightly triangular, with narrow, acute colpi.
Fagus	3	exine ornamented, granulate, big pollen (45-52 µm)
Ambrosia	3	exine ornamented, with echini, furrows very short, inconspicuous
Artemisia	3	furrows well defined
Asteraceae	3	exine ornamented, with echini
Fabaceae	3	reticulate exine, isopolar
Aesculus	3	striate exine , prolate
Vitis	3	reticulate exine, isopolar, spheroidal, fine mesh (<1 um)
Ligustrum	3	reticulate exine, with margo, isopolar, spheroidal, muri with gemmae, inconspicuous pores
Sambucus	3	reticulate exine, isopolar, spheroidal
Hedera	3	reticulate exine, with margo, isopolar, spheroidal
Tilia	3	reticulate exine, isopolar, oblate, thickening of endexine
Polygonaceae	3-4	exine ornamented, granulate
Boraginaceae	>5	stephanocolporate

onci: lens-shaped structure beneath the aperture

vestibulum: exine thickened around pori forming shield-shaped areas (aspidote)

operculum: thick exine membrane covering an aperture

arci: thickenings on the pollen wall between pores forming curves from one aperture to another

margo: mesh decreasing when bordering the aperture (furrow)

oblate: pollen grain with a polar axis shorter than the equatorial diameter

prolate: pollen grain with a polar axis longer than the equatorial diameter

reticulate: sexine (upper layer of the exine) sculpturing with reticulum, a network like pattern formed by system of ridges (muri)

verrucate: sexine sculpturing with wart-like elements

striate: sexine sculpturing with elongated elements, predominantly parallel arranged

gemmate: sexine sculpturing with globular exine element more than 1 µm in diameter (gemmae)

granulate: sculpture element of different size and shape; smaller than 1 µm

echinate: pointed ornamentation element longer and/or wider than 1 µm (echini)

Appendix 2 Indicative metabarcoding surveys for diversity assessment grouped by environment and for each environment by year.

Παράρτημα 2 Ενδεικτικές έρευνες metabarcoding για εκτίμηση βιοποικιλότητας ομοδοποιημένες ανά περιβάλλον και για κάθε περιβάλλον με χρονολογική σειρά.

Study	Type of sample	Target organism	Genetic marker
Air			
Nunez et al. 2017	tape (Burkard trap)	pollen, fungal spores, bacteria	ITS2, 16S, ITS-4
Kraaijeveld et al. 2015	tape (Burkard trap)	pollen	trnL (c-h)
Yooseph et al. 2013	air filter (wet cyclone)	bacteria	16S
Tringe et al. 2008	air filter	bacteria	16S
Terrestrial			
Bell et al. 2017	insect-collected pollen	pollen	rbcL, ITS2
Pornon et al. 2016	insect-collected pollen	pollen	trnL, ITS1
Smart et al. 2016	insect-collected pollen	pollen	ITS
McFrederick et al. 2016	insect-collected pollen	pollen	rbcL
Richardson et al. 2015a	insect-collected pollen	pollen	ITS2
Richardson et al. 2015b	insect-collected pollen	pollen	ITS2, matK, rbcL
Keller et al. 2015	insect-collected pollen	pollen	ITS2
Sickel et al. 2015	insect-collected pollen	pollen	ITS2
de Vere et al. 2017	honey	pollen	rbcL
Laha et al. 2017	honey	pollen	rbcL, matK, ITS2
Hawkings et al. 2015	honey	pollen	rbcL
Yoccoz et al. 2012	soil	plants	tranL (g-h)
Taberlet et al. 2012	soil	plants	tranL (g-h)
Andersen et al. 2012	soil	vertebrates	16S
Bienert et al. 2012	soil	earthworms	16S
Geml et al. 2009	soil	fungi	ITS
Zinger et al. 2009	soil	bacteria, fungi	16S, ITS1
Calvignac et al. 2012	carrion flies	mammals	16S, 12S
Hiiesalu et al. 2012	roots	plants	trnL (c-d)
Soininen et al. 2015	feces	plants	trnL (g-h), trnL (c-h)
Shrivathsan et al. 2014	feces	plants	trnL (g-h)
Czernik et al. 2013	feces	plants	trnL (g-h)
Ando et al. 2013	feces	plants	trnL (g-h)
De Barba et al. 2013	feces	plants	trnL (g-h), ITS1, ITS2
Baamrane et al. 2012	feces	plants	trnL (g-h)
Kowalczyk et al. 2011	feces	plants	trnL (g-h)
Raye et al. 2011	feces	plants	trnL (g-h)
Pegard et al. 2009	feces	plants	trnL (g-h)
Valentini et al. 2008	feces	plants	trnL (g-h)
Shehzad et al. 2012	feces	vertebrates	12S
Freshwater			
Yamamoto et al. 2017	water	fish	12S
Lim et al. 2016	water	metazoa	COI
Evans et al. 2016	water	fish/amphibians	16S, 12S
Gibson et al. 2015	benthos	macroinvertebrates	COI
Lallias et al. 2015	benthos	meiofauna	nSSU

Appendix 2 (continued)

Study	Type of sample	Target organism	Genetic marker
Freshwater			
Stoof-Leichsenring et al. 2012	lake	diatoms	rbcL
Hahibabei et al. 2011	benthos	macroinvertebrates	COI
Marine			
Sogin et al. 2009	water	bacteria	16S
Barberàn et al. 2012	water	bacteria, archae	16S
Leray & Knowlton 2015	benthos	invertebrates	COI
Borrell et al. 2017	water	invertebrates	COI, 18S

Appendix 3 Protocol sheet for pollen preparation and next generation sequencing.

Παράρτημα 3 Πρωτόκολλο για την προετοιμασία γύρης και αλληλούχιση.

Reagents, kits and other materials used

Nucleomag 96 Plant kit, (Macherey-Nagel, Düren, Germany)

Go Taq PCR kit (Promega, Madison, WI, USA)

MineElute Gel Purification kit (Qiagen, GmbH, hilden, Germany)

TruSeq DNA sample preparation kit V2 (Illumina, CA, USA)

Lycopodium tablets (Batch 3862, Lund University, Lund, Sweden)

Mixed cellulose ester filter, 5μm, 47 mm (Merck Millipore Ltd, Cork, Ireland)

Metal mesh sieve, 200 μm (Retsch, Haan, Germany)

Filtration system

Tauber bottles (5 l), collars and mesh net

Ethanol, Glycerol, Phenol

Glass tubes (15 ml)

Instruments

Kingfisher (Thermo Fisher Scientific, Waltham, MA, USA)

Veriti 96 well thermal cycler (Applied Biosystems, Foster City, CA, USA)

Qiaxcel (Qiagen, GmbH, Hilden, Germany)

Qubit (Life Technologies, Thermo Fisher Scientific Inc.)

Optical microscope (Leitz Diaplan, Ernst Leitz Wetzlar, Wetzlar, Germany)

Retsch MM200 mixer mill (Retsch, Haan, Germany)

Sample Preparation

Prepare Tauber traps with 700 mL of 1 Glycerol: 1 Water : 1 Ethanol + 2 g l^{-1} Phenol

Filtration

Microscopy: Dissolve Lycopodium tablet(s) with distilled water in a magnetic agitator and add the dissolved *Lycopodium* in the trap

Pre-filter the collected samples with a sieve of 200 μm in an 1 l becker.

Filter the sample into with a 5 μm filter with filtration system of 1 l capacity.

Insert the filter in a labeled small petri dish and dry it in the oven 65°C for 2-3 hours.

After the filter is dry, either immediately process or close with parafilm and store at -20°C.

Place it in a glass tube (15 ml) (Unfreeze first if the filter is stored at -20°C)

Appendix 3 (continued)

Add 5 ml of acetone, vortex for 2-3 min (filters should get very well dissolved).

Centrifuge 3 min, 2,300 rpm, discard the supernatant and add 1 ml of acetic acid to the pellet, vortex for 2-3 min.

Centrifuge 3 min, 2,300 rpm, discard the supernatant and add 2 ml of distilled water, plus one drop of ethanol to reduce the surface tension.

Repeat the washing for two more steps.

Add 1 ml of distilled water, transfer into 2 ml tubes and centrifuge for 3 min at 13,500 rpm

Discard the supernatant and store at -20°C.

Microscopy: 0.5 ml of the supernatant kept, two drops of glycerol added and the samples stored at 4°C.

Microscopy: transfer a small aliquot of the pellet on a microscope slide and color with basic fuchsine. Analyze a minimum of 400 pollen grains.

DNA extraction

Unfreeze the samples and put the Kingfisher plates under UV

In 2 ml tubes, add : steel bead, 500 µl Lysis buffer, 10 µl RNase and close with parafilm.

Put the tubes in the adaptors of the mixer mill and grind for 1 min at 30 Hz, in two steps.

Incubate for 30 min at 56°C.

Turn on Kingfisher machine and select the protocol (KF-Genomic DNA from Plant).

Prepare the plates for Kingfisher by following the instructions of Nucleomag kit.

Load to the machine the first plate, continue with the next position and the second plate, etc.

PCR amplification

PRC room: Pollen DNA ισ amplified in 3 separate reactions with the universal chloroplast c-A49325 and h-B49466 *trn*L primers and the Go Taq protocol (In case of no amplification, we diluted the samples (usually 1:20)

Post PCR room: Pool together the 3 reactions

The final product is gel purified using the MinElute Gel Purification kit (Qiagen) and eluted in 53 µl

Quantification of the PCR product with Qubit 2.0 Fluorometer

Library preparation

100 ng of the purified PCR product for library (TruSeq DNA sample preparation kit V2)

Pool together the libraries in equimolar ratio

Sequenced using MiSeq Reagent Kit v3 in Illumina MiSeq platform

Appendix 4 Taxa of interest for the construction of the reference database. The taxa are given in alphabetical order. For the pollination period the numbers 1-3 correspond to months of the year, in which the plants are recorded in aerobiological data at the Aerobiological Monitoring Centre of Fondazione Edmund Mach in San Michele All' Adige (1: January-February, 2: March-August, 3: September-December). For the pollination mode, A: anemophilous, E: entomophilous (after Lewis 1983)). For the growth form, W: woody, H: herbaceous (after Tutin et al. (1968–1980, 1993), Lewis et al. (1983)). The families are given in alphabetical order.

Παράρτημα 4 Τάξα ενδιαφέροντος για την κατασκευή βάσης δεδομένων αναφοράς. Τα τάξα δίνονται κατά αλφαβητική σειρά. Για την περίοδο επικονίασης, τα 1-3 αντιστοιχούν σε μήνες του χρόνου, στους οποίους τα φυτά βρέθηκαν σε αεροβιολογικά δεδομένα του σταθμού του Fondazione Edmund Mach στο San Michele All' Adige (1: Ιανουάριος-Φεβρουάριος, 2: Μάρτιος-Αύγουστος, 3: Σεπτέμβριος-Δεκέμβριος). Ως προς τον τρόπο επικονίασης, A: ανεμόφιλα, E: εντομόφιλα (σύμφωνα με Lewis 1983). Ως προς την αυξητική τους μορφή, W: ξυλώδη, H: ποώδη (σύμφωνα με Tutin et al. (1968–1980, 1993), Lewis et al. (1983)). Οι οικογένειες δίνονται με αλφαβητική σειρά.

Family	Genus	Species	Common name	Pollination period	Pollination mode	Growth form
Aceraceae	*Acer*		maple	2	A, E	W
Amaranthaceae			amaranth	2	A, E	H
Anacardiaceae	*Pistacia*		pistachio	2	A	W
Apiaceae			parsley, carrot	2	E	H
Araliaceae	*Hedera*		ivy	3	E	W
Asteraceae			daisy, sunflower	2	A, E	H
Asteraceae	*Ambrosia*		ragweed	2	A	H
Asteraceae	*Artemisia*		mugwort	2	A	H
Betulaceae	*Betula*		birch	1	A	W
Betulaceae	*Alnus*		alder	1	A	W
Boraginaceae			borage, forget-me-not	2	E	H
Brassicaceae			crucifer, cabbage	2	E	H
Buxaceae	*Buxus*		box	2	E	W
Campanulaceae			bellflower	2	E	H
Cannabaceae	*Humulus*		hop	2	A	H
Caprifoliaceae	*Sambucus*		elder	2	E	W
Caryophyllaceae			carnation, campion	2	E	H
Chenopodiaceae			goosefoot	2	A, E	H
Corylaceae	*Carpinus*	*Carpinus betulus*	hornbeam	2	A	W
Corylaceae	*Corylus*	*Corylus avellana*	hazel	1, 2	A	W
Corylaceae	*Ostrya*	*Ostrya carpinifolia*	hop-hornbeam	2	A	W
Cupressaceae			cypress	1, 2	A	W
Taxaceae			yew	2	A	W
Cyperaceae			sedge	2	A	H
Ericaceae			heath	1, 2	A, E	W
Euphorbiaceae			spurge	2	E	H, W
Fabaceae			legumes	2	E	H, W
Fagaceae	*Castanea*		chestnut	2	A,E	W
Fagaceae	*Fagus*		beech	2	A	W
Fagaceae	*Quercus*		oak	2	A	W
Ginkgoaceae	*Ginkgo*	*Ginkgo biloba*	ginkgo	2	A	W
Poaceae			grasses	2	A	H
Juglandaceae	*Juglans*		walnut	2	A	W
Juncaceae			rush	2	A	H

Appendix 4 (continued)

Family	Genus	Species	Common name	Pollination period	Pollination mode	Growth form
Lauraceae	*Laurus*	*Laurus nobilis*	laurel	2	E	W
Moraceae			mulberry	2	E	W
Oleaceae	*Fraxinus*	*Fraxinus excelsior*	common ash	2	A	W
Oleaceae	*Fraxinus*	*Fraxinus ornus*	flowering ash	2	E	W
Oleaceae	*Ligustrum*		privet	2	E	W
Oleaceae	*Olea*	*Olea europaea*	olive	2	A	W
Oleaceae			jasmin, forsynthia	2	E	W
Palmaceae			palm	2	A	W
Papaveraceae	*Papaver*		poppy	2	E	H
Pinaceae	*Abies*		fir	2	A	W
Pinaceae	*Cedrus*		cedar	3	A	W
Pinaceae	*Larix*		larch	2	A	W
Pinaceae	*Picea*		spruce	2	A	W
Pinaceae	*Pinus*		pine	2, 3	A	W
Plantaginaceae	*Plantago*		plantain	3	A	H
Platanaceae	*Platanus*		plane	2	A	W
Polygonaceae	*Rumex*		sorrel	2	A, E	H
Rosaceae			rose, apple, almond	2	A, E	H, W
Rubiaceae			bedstraw	2	E	H
Salicaceae	*Populus*		poplar	2	A	W
Salicaceae	*Salix*		willow	2	A, E	W
Sapindaceae	*Aesculus*		horse chestnut	2	E	W
Saxifragaceae			saxifrage	2	E	H
Simaroubaceae	*Ailanthus*	*Ailanthus altissima*	heaven tree	2	A	W
Tiliaceae	*Tilia*		linden	2	E	W
Ulmaceae	*Ulmus*		elm	2	A	W
Urticaceae	*Parietaria*		sticky-weed	2, 3	A	H
Urticaceae	*Urtica*		nettle	2, 3	A	H
Vitaceae	*Vitis*		grapevine	2	A	W

Appendix 5 Taxa identified by use of next generation sequencing (NGS) for the three sampling periods and overall, and also after the microscopic method for one period and their relative abundance. For the NGS dataset, given is also the number of reads and the number of samples, in which each taxon was observed.

Παράρτημα 5 Τάξα που αναγνωρίστηκαν με αλληλούχιση νέας γενιάς (NGS) για τρεις δειγματοληπτικές περιόδους και συνολικά, και με τη μέθοδο μικροσκοπίας για μία περίοδο και οι σχετικές τους αφθονίες. Για τα δεδομένα της NGS, δίνεται επίσης ο αριθμός των αλληλουχιών και ο αριθμός των δειγμάτων στα οποία παρατηρήθηκε το κάθε τάξο.

| | | | | Next Generation Sequencing | | | | | | | | | | | | Microscope | |
Growth Form	Family	Genus	Species	October 2014-March 2015 No. reads	No. samples	% abundance	March-July 2015 No. reads	No. samples	% abundance	July-October 2015 No. reads	No. samples	% abundance	October 2014-October 2015 No. reads	No. samples	% abundance	March-July 2015 Taxa	% abundance
Trees and shrubs	Aceraceae	Acer	A. pseudoplatanus	137	1	0.0042	105	6	0.0025	10830	1	0.2941	11072	8	0.0994		
	Anacardiaceae	Pistacia	P. terebinthus†	926	1	0.0282	3	2	0.0001	0	0	0.0000	929	3	0.0083		
	Betulaceae	Alnus	A. viridis	108987	17	3.3154	4454	18	0.1069	206490	13	5.6065	319931	48	2.8726	Alnus	2.3361
			Alnus sp.	1638	17	0.0498	65	7	0.0016	21	4	0.0006	1724	28	0.0155		
																Betula	0.7268
	Caprifoliaceae	Lonicera	L. xylosteum	0	0	0.0000	753	1	0.0181	0	0	0.0000	753	1	0.0068	Lonicera	0.0649
			Lonicera sp.	2	2	0.0001	118	3	0.0028	897	1	0.0244	1017	6	0.0091		
		Sambucus	Sambucus sp.	238	7	0.0072	69	9	0.0017	4154	3	0.1128	4461	19	0.0401	Sambucus	0.0130
	Corylaceae	Corylus/Ostrya	C. avellana/O. carpinifolia/	323530	18	9.8419	1551	18	0.0372	227612	16	6.1800	552693	52	4.9626	Corylus	0.0260
		Carpinus	C. betulus													Carpinus	0.0389
																Ostrya	2.5438
	Cupressaceae	Cupressus	C. arizonica†	15320	18	0.4660	43	9	0.0010	4060	3	0.1102	19423	30	0.1744	Cupressaceae-Taxaceae	1.2200
			C. sempervirens†	56020	18	1.7041	66	14	0.0016	10	4	0.0003	56096	36	0.5037		
		Juniperus	J. communis	76386	18	2.3237	5105	18	0.1225	1167	9	0.0317	82658	45	0.7422		
			J. sabina†	27337	18	0.8316	3825	18	0.0918	2123	3	0.0576	33285	39	0.2989		
		Taxodium	T. distichum†	3943	15	0.1199	1	1	0.0000	0	0	0.0000	3944	16	0.0354		
		Thuja	T. occidentalis†	12572	9	0.3824	3	3	0.0001	0	0	0.0000	12575	12	0.1129		
			T. orientalis†	11592	17	0.3526	2	2	0.0000	1	1	0.0000	11595	20	0.1041		
	Taxaceae	Taxus	T. baccata	68445	17	2.0821	6	5	0.0001	5	4	0.0001	68456	26	0.6147		
	Ericaceae	Kalmia	K. procumbens	562	8	0.0171	454	5	0.0109	5291	3	0.1437	6307	16	0.0566	Ericaceae	0.2077
		Vaccinium	V. myrtillus	1	1	0.0000	5595	1	0.1343	0	0	0.0000	5596	2	0.0502		
	Fabaceae	Indigofera	I. circinella†	295	3	0.0090	0	0	0.0000	0	0	0.0000	295	3	0.0026	Fabaceae	0.1298
	Fagaceae	Quercus	Quercus sp.	1745	15	0.0531	3665	18	0.0880	1354	9	0.0368	6764	42	0.0607	Quercus	0.7917
																Castanea	0.1817
																Fagus	0.0260
	Ginkgoaceae	Ginkgo	G. biloba†	3217	3	0.0979	14	3	0.0003	0	0	0.0000	3231	6	0.0290		
	Juglandaceae	Juglans	J. regia	307	2	0.0093	407	12	0.0098	0	0	0.0000	714	14	0.0064	Juglans	0.2077
			J. nigra	215	1	0.0065	0	0	0.0000	0	0	0.0000	215	1	0.0019		
	Moraceae	Broussonetia	B. papyrifera†	472	7	0.0144	38	3	0.0009	0	0	0.0000	510	10	0.0046		
	Oleaceae	Fraxinus/Olea/Syringa	Fraxinus sp./O. europaea/	22460	17	0.6832	296	14	0.0071	15	3	0.0004	22771	34	0.2045	F. excelsior	0.1947
			S. vulgaris													F. ornus	4.0883
																O.europaea	0.0779
																Oleaceae	1.1162

131

Appendix 5 (continued)

Next Generation Sequencing

Growth Form	Family	Genus	Species	October 2014–March 2015			March–July 2015			July–October 2015			October 2014–October 2015		
				No. reads	No. samples	% abundance	No. reads	No. samples	% abundance	No. reads	No. samples	% abundance	No. reads	No. samples	% abundance
	Pinaceae	Abies	Abies sp.	10095	18	0.3071	588865	18	14.1320	75	18	0.0020	599035	17	5.3787
		Cedrus	C. deodara†	1329	11	0.0404	221	18	0.0053	735	13	0.0200	2285	42	0.0205
			Cedrus sp.†	179668	18	5.4655	447	18	0.0107	1199963	18	32.5810	1380078	54	12.3916
		Larix	L. decidua	1492182	18	45.3926	51779	18	1.2426	66811	18	1.8140	1610772	54	14.4630
		Picea	Picea sp.	291686	18	8.8732	675147	18	16.2027	326558	18	8.8666	1293391	54	11.6133
		Pinus	Pinus sp.	450127	18	13.6930	2722746	18	65.3426	930308	18	25.2594	4103181	54	36.8422
	Platanaceae	Platanus	Platanus sp.†	2265	12	0.0689	22	4	0.0005	0	0	0.0000	2287	16	0.0205
	Rosaceae	Prunus	Prunus sp.	40	3	0.0012	892	6	0.0214	0	0	0.0000	932	9	0.0084
	Salicaceae	Salix	Salix sp.	3076	11	0.0936	3	3	0.0001	0	0	0.0000	3079	14	0.0276
		Populus	Populus sp.	12921	17	0.3931	13	5	0.0003	0	0	0.0000	12934	22	0.1161
	Tiliaceae	Tilia	Tilia sp.	547	4	0.0166	311	2	0.0075	0	0	0.0000	858	6	0.0077
	Ulmaceae	Ulmus	Ul. glabra	33665	14	1.0241	71	4	0.0017	0	0	0.0000	33736	18	0.3029
Herbs	Apiaceae	Daucus	Daucus sp.	1	1	0.0000	46	2	0.0011	12005	1	0.3260	12052	4	0.1082
	Asteraceae	Ambrosia/Taraxacum/others	Ambrosia sp./T. officinale/others	1288	6	0.0392	6487	13	0.1557	10028	5	0.2723	17803	24	0.1599
		Cirsium	Cirsium sp.	75	5	0.0023	367	9	0.0088	8954	4	0.2431	9396	18	0.0844
		Senecio/Jacobaea	Senecio sp./Jacobaea sp.	21	4	0.0006	162	7	0.0039	12665	3	0.3439	12848	14	0.1154
	Brassicaceae														
	Campanulacea	Campanula	C. rotundifolia	0	0	0.0000	503	1	0.0121	0	0	0.0000	503	1	0.0045
	Cannabaceae	Humulus	H. lupulus	0	0	0.0000	0	0	0.0000	15941	3	0.4328	15941	3	0.1431
	Caryophyllace.	Silene	S. vulgaris	0	0	0.0000	31	4	0.0007	6827	2	0.1854	6858	6	0.0616
	Chenopodiace.	Chenopodium	C. album	2	2	0.0001	3	3	0.0001	64342	8	1.7470	64347	13	0.5778
	Fabaceae	Medicago	M. sativa	508	4	0.0155	6	2	0.0001	0	0	0.0000	514	6	0.0046
	Hypericaceae	Hypericum	H. perforatum	1	1	0.0000	270	2	0.0065	245	1	0.0067	516	4	0.0046
	Lamiaceae														
	Plantaginaceae	Plantago	P. lanceolata	106	1	0.0032	275	11	0.0066	80	2	0.0022	461	14	0.0041
	Polygonaceae														
	Ranunculaceae	Ranunculus	R. bulbosus	151	3	0.0046	695	13	0.0167	0	0	0.0000	846	16	0.0076
	Rosaceae	Potentilla	P. palustre	966	5	0.0294	2	1	0.0000	7	1	0.0002	975	7	0.0088
	Rubiaceae														
	Scrophulariaceae														
	Urticaceae	Parietaria	P. judaica	2	2	0.0001	265	10	0.0064	29062	6	0.7891	29329	18	0.2633
		Urtica	U. dioica	164	8	0.0050	3385	18	0.0812	310326	15	8.4259	313875	41	2.8183
Graminoids	Cyperaceae														
	Juncaceae	Luzula	Luzula sp.	28	2	0.0009	569	11	0.0137	1	1	0.0000	598	14	0.0054
		Juncus	Juncus sp.	1	1	0.0000	539	10	0.0129	0	0	0.0000	540	11	0.0048
	Poaceae	Setaria/Phragmites	S. verticillata/P. australis	280	3	0.0085	82	6	0.0020	4524	2	0.1228	4886	11	0.0439
		Alopecurus/Phleum	A. pratensis/Phleum sp.	63	3	0.0019	13666	16	0.3280	47	4	0.0013	13776	23	0.1237
		Avenella	A. flexuosa	259	5	0.0079	19201	17	0.4608	20246	6	0.5497	39706	28	0.3565
		Brachypodium	B.sylvaticum	8	5	0.0002	11536	13	0.2769	42	3	0.0011	11586	21	0.1040
		Calamagrostis	C. epigeos	3	2	0.0001	2092	15	0.0502	34532	3	0.9376	36627	20	0.3289
		Dactylis	D. glomerata	299	5	0.0091	4584	15	0.1100	11	2	0.0003	4894	22	0.0439
		Festuca/Trisetum/Lolium	Festuca sp./T. spicatum/Lolium sp	5346	14	0.1626	28450	18	0.6828	26641	7	0.7233	60437	39	0.5427
		Poa	Poa sp.	85	10	0.0026	434	14	0.0104	1617	5	0.0439	2136	29	0.0192
	Unidentified			63678	18	1.9371	6072	18	0.1457	136395	16	3.7033	206145	52	1.8510
	Total			2550936		77.6001	4140239		99.3607	3218988		87.4008	9910163		88.9827

Microscope March–July 2015

Taxa	% abundance
Abies	1.1551
Cedrus	0.0519
Larix	0.5970
Picea	8.0208
Pinus	37.9883
Pinaceae	0.4802
Rosaceae	0.1817
Salix	0.0519
Tilia	0.0649
Ulmus	0.0130
Vitis	0.0130
Apiaceae	0.2726
Taraxacum/others‡	0.2466
Cirsium/others‡	0.0649
Helianthus/Senecio/others‡	0.0779
Artemisia	0.0260
Asteraceae	0.2596
Brassicaceae	0.0519
Campanulaceae	0.0389
Cannabaceae	0.0130
Caryophyllaceae	0.0519
Chenopodiaceae-Amaranthaceae (see Rosaceae above)	0.0779
Lamiaceae	0.0130
Plantago	0.5451
Polygonaceae	0.4153
Ranunculaceae (see Fabaceae above)	0.1557
Rubiaceae	0.0130
Scrophulariaceae	0.0649
Urticaceae	0.6100
Cyperaceae	0.6360
Juncaceae	1.1162
Poaceae	20.9215
Unidentified	11.7197
Total	86.0091

† Taxa that are not found in the plant checklist of the Park (Festi & Prosser 2000)

‡ Asteraceae pollen grains are grouped according to Bucher et al. (2004)

132
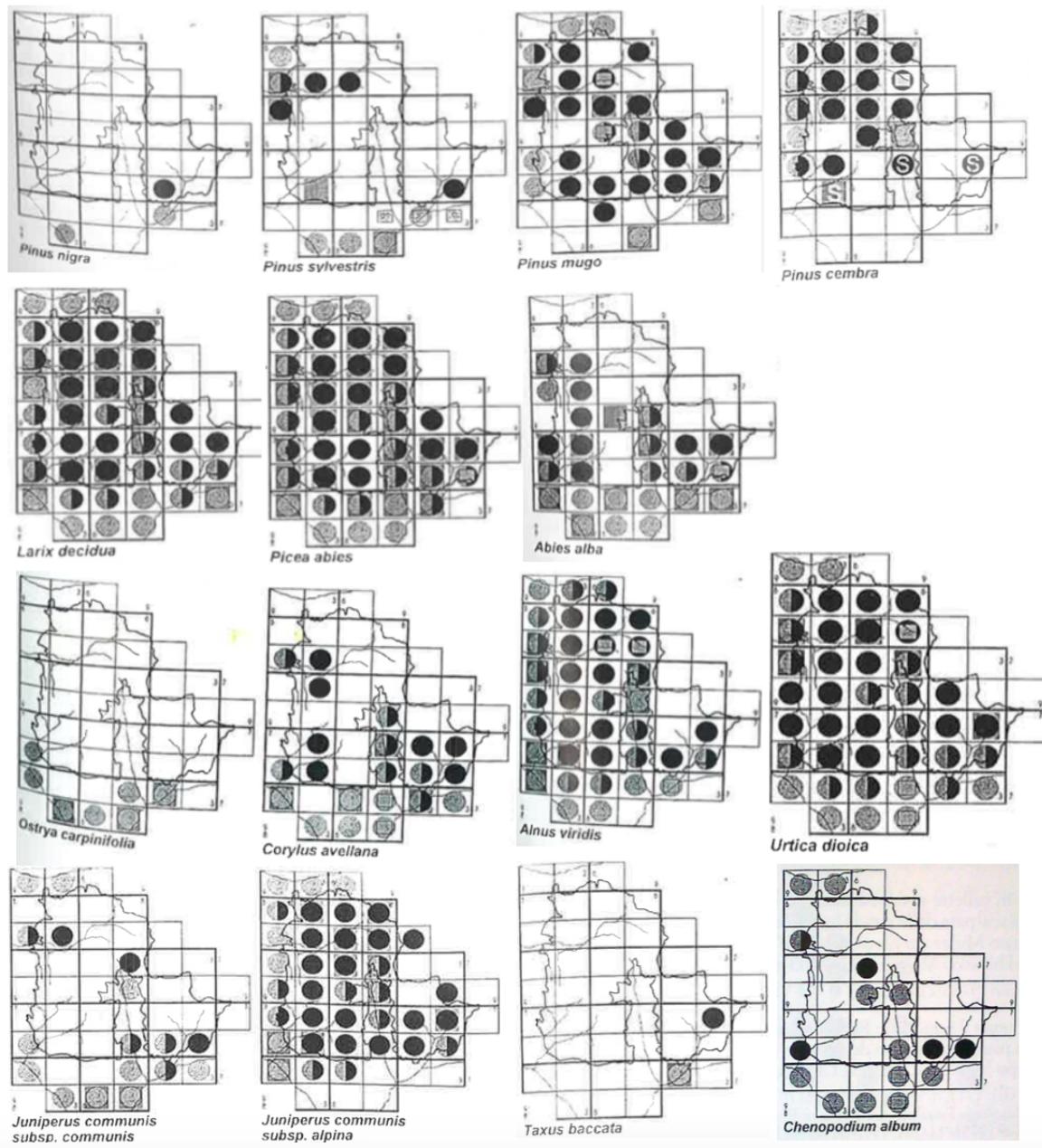
Appendix 6 Transects that give with a circle the presence of the main contributing pollen taxa in the area of the park (black circle) and out of it (grey circle) (Festi and Prosser 2000)

Παράρτημα 6 Διατομές που δίνουν με κύκλο την παρουσία των κύριων taxa γύρης στην περιοχή του πάρκου (μαύρος κύκλος) και έξω από αυτό (γκρι κύκλος) (Festi and Prosser 2000)