Loop-Mediated Isothermal Amplification for the Mapping of Microbes: An Anti-Capitalist Approach

By

Ali Bektaş Tonak

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor in Philosophy

in

Environmental Science, Policy and Management

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Ignacio H. Chapela, Chair
Professor Laura Nader
Professor George K. Roderick

Spring 2015

Abstract

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The field of microbial ecology is hindered by our general inability to map microbes on a geographical scale. This is in part due to technical limitations posed by current DNA detection reactions. This dissertation presents the application of Loop-Mediated Isothermal Amplification (LAMP) for the purpose of amplifying DNA from pollen grains; down to a single grain and with a resulting fluorescence signal to indicate the presence or absence of the particular DNA fragment in question. Three academic papers outlining this method comprise the bulk of the work and are bookended by a brief survey of microbial detection efforts to date and future applications of our method.

Beyond technical limitations there are social and political priorities embedded within the biological sciences that are implicit in our inability to map microbes. Therefore, any advance in the resolution of microbial mapping must also challenge these priorities. In place of the centralized research conducted by experts in universities and corporations, and ultimately for the purpose of capital accumulation, we propose a distributed and horizontally organized mapping network antagonistic to capitalist biology.

Pollen grains are our choice of microbe because they are the vector of reproduction for flowering plants and thus an important source of contamination of transgenic DNA from transgenic plants. The growing rejection of transgenic crops across the globe points to a strong desire to identify their location, utilizing microbial mapping methods, and eliminate their spread.

The technical advances presented here, alongside our social and political commitments, aim to assist in that effort.

For my mother,
who made me who I am and continues to be my brightest inspiration.

Contents

Acknowledgements

Dozens of people around the world have played a key role in making the work presented in the following pages a reality and continue to support it in a variety of ways. The list is large and surely I will be omitting some, I hope I am forgiven in advance.

I must express my gratitude for the Genøk team in Tromsø, Norway for providing a much-needed refuge away from the Bay where a significant amount of the research was conducted and its general direction defined. Much thanks to Terje Traavik, Thomas Bøhn, Frøydis Gillund, Connie Tümmler, Julia Eggert and David Quist.

In Mexico, where I spent close to 2 years conducting field work and working at the Instituto de Ecología at UNAM, I couldn't have done it without Antonio Serratos, Javier Serramiento, Alma Piñeyro-Nelson and of course Elena Alvarez-Buylla who supported me materially by graciously hosting me at her lab and, even more importantly, intellectually by always having a open door to discuss the various challenges presented by the Mexican landscape.

Recent friends and collaborators in Mexico, Rémy Vandame and Eric Vides and in Spain, Rosa Binimelis and Jaoquin Arqué Villar, have opened up the world of honey to our laboratory. Much thanks to them with great anticipation for the future.

David Noble remains a powerful figure for me as he stands over the machines of biotechnology holding the hammer of Ned Ludd between his hands, sadly our budding friendship was cut short by his untimely death.

Cristian Orrego gave essential guidance during his nighttime visits to our lab.

Lisa Thompson has been a co-conspirator for the past decade and has shared many wine-infused nights of laughter.

Kelly Gawel was present with her love and support for the most important periods of this work, I can't thank her enough for being there, continuing to be and for being patient with me.

My comrades here in the Bay and around the world, who shall remain nameless as they are behind their masks, thank you for creating that which truly matters. Let's never give up until we are all free.

Maria, we met as I was preparing this manuscript and the joy of being with you made it much less of a burden. Excitement, adventure is ever-present with you, all of the time. Can't wait for everything!

Lastly but most importantly: Ignacio. This is our work together. You have been a mentor, a comrade and a friend. We've had many ups and downs throughout the years and it feels like there is finally light at the end of the tunnel. I can't think of a better partner-in-crime. Cheers to the future!

May 2015, Oakland CA

Chapter 1:
Introduction

There is a dialectical relationship between the development of technical instrumentation and the production of knowledge through scientific investigation. This dialectics is not simple: certain seemingly contradictory positions do not merely inform each other but instead, there is an opening up of certain worlds to our gaze while others are shuttered in the process. A further dimension concerns the actors who use these instruments and to what ends and with what mandate. Those who are in positions of power to direct scientific activities (specialized know-how, laboratory facilities and budgets) lay claim of independence and objectivity by invoking a pursuit of unadulterated and absolute knowledge. This power, coded in precise measurements, arcane descriptions and manipulation is best placed in the social and political sphere; it shapes the world we inhabit, our daily lives and our biology. When one speaks of the technics of scientific inquiry one must speak of sociopolitical dialectics (Levins, 1985).

The work presented in the following pages aims to explicitly engage with the social and political spheres and not pretend to contain a false objectivity. Those of us conducting this research do so guided by locating our understanding of Biology as part of the positions we have chosen to take in society and politics. In doing this, we also stand counter to the understanding of Biology as a discipline enabling the commodification of life, the creation of industrial processes overtaking the natural world and alienating humans from non-human life. Our wish is for a revolutionary biology in solidarity with resistance movements struggling to overthrow the existing global economy. We see our research as a contribution to these movements by facilitating a deeper understanding of life and its processes.

Instruments Gazing into the Invisible

The visualization of invisible worlds, the macroscopic yet too far, and the close yet microscopic ones, permitted the modern sciences to advance leaps and bounds. Lens-crafting and the development of the telescope and microscope are the initial technical pillars onto which this visualization was built upon (Bradbury, 1968; Wilson, 1997a). The history of the development of these two instruments is illuminating as they were initially conceived much more as beautiful objects acting as parlor tricks for the bourgeoisie in 17th century Europe rather than primarily for assisting the natural philosophers of that time (Bennett, 1989). Beginning with Galileo and Leewenhoek their use became much more professionalized and gradually isolated from society in general, continuing to this day with the deployments of space telescopes and the development of scanning electron microscopes.

The severing of peoples from the instruments of investigation has been integral to the creation of a scientific class of elites who determine which questions will be

pursued in the lab and field, which answers are deemed appropriate ones and even more fundamentally who will be admitted into their cadre to pursue these endeavors. In fact, certain research units are merely driven by the simple processing of samples through high-tech instruments. Today, DNA sequencing is the most obvious example of this kind of instrument-driven research (Schuster, 2008). A process of investigation and inquiry into the natural world which acts counter to the hegemonic power structures of today, namely against capitalism and the state-form of organizing society must ultimately possess the technical instruments necessary to do so. From the beginning, these instruments must also be conceived and conceptualized with these ends in mind.

Detecting Microbial Life

If we have a certain political position, which is informed by, and informs, our analysis of the social and biological, we must develop technics and instruments in accordance with it. What would microbial investigations countering capitalist biology look like? And for the purposes of this dissertation, acknowledging a trialectical understanding of the relationship between science, its instruments and it's users, the central question becomes: **how should the instruments of anti-capitalist microbial ecology research operate, who are its users and how would it influence them?**

In terms of immediate social consequences, seeing and understanding microbial life is totally disproportionate to the actual size of the microbes. Comprising the majority of the earth's biota both in terms of biomass and species diversity (Gould, 1996) microbes permeate every aspect of life from our bodies to the far reaches of the atmosphere. Additionally, having preceded all other life-forms they also provide a vast amount of clues to our evolutionary histories and futures (Margulis, 1986). Microbes and their unique processes are also what the edifice of the hegemonic field of genetics has been based on (Nicholl, 2008). Perhaps nothing is more important in biology than what can be observed through a microscope.

Images we receive through the ocular are timeless and static ones, mostly of individuals. They obscure the processes, flows, shifts, and transitions and thus prevent an understanding of the dynamic nature of life. For the period immediately surrounding the microscope's inception it was the perfect instrument allowing Hooke and Leewenhoek to meticulously sketch out the anatomical detail of lice, protozoa, embryos and almost anything they could fit on a slide and focus on (Hooke, 1667; Leeuwenhoek and Hoole, 1816). The unraveling of this invisible world eventually led to the observation of chromosomes, their replication/segregation during cell division and brought about the field of genetics as we know today.

But the contemporary research agenda is vastly different from the late 17^{th} and 18^{th} century where the main emphasis was awarded to describing previously unknown forms. Today, everyone from ivory tower research scientists to mobilized amateurs are trying to focus their attention on the global scale to observe and theorize about

ecological processes and changes. The fashionable status of all things related to the climate is the clearest example of this trend. But beyond that, the current phase of capitalism, globally intertwined and organized logistically on such a scale requires an equally intertwined and organized biological investigative effort in order to assist in bringing about its downfall. What would investigations of shifts and flows of microbial life on a global scale look like?

The answer to this question from the scientific elite is wholesale sampling and DNA sequencing. The Human Genome Project was merely the opening (and failed) act of sequence fetishism (Latham, 2011), and people ranging from those integral to that effort, such as Craig Venter, to numerous bioinformatics projects are attempting to rectify its shortcomings not by reconsidering the central paradigm of sequencing but by attempting to sequence as many individuals from as many species as possible.

Mapping the Movement of Transgenic Organisms

Transgenic organisms are life-forms which contain chimeric DNA sequences, conceived of and crudely introduced into nuclei, chloroplasts or mitochondria in a laboratory. Since the first environmental release of a transgenic organism, the ice-minus bacteria, into the world from Berkeley, California in 1987 (Kibby, 1987) (Seidler and Hern, 1988) and especially after the rapid ascendency of the commercial cultivation of genetically modified organisms (GMOs) through the 90s there has been an accompanying and as rapidly increasing outrage at their global propagation. Contamination of seed stocks, food products and non-GMO farms and fields by transgenic DNA has frustrated both concerned citizens as well as those with economic interests on all sides of the controversy. The concern lies in part on the still fully unexplored effects on human health, the ecosystems GMOs are cultivated in and how it enables transnational corporations to exert further control on global agriculture systems. This frustration is also fueled by the near impossibility to effectively detect transgenic pieces of DNA as they move from their initial points of cultivation in the environment to other secondary zones and onward.

The Case of Transgenic Contamination of Corn in Mexico

Corn is more than a staple crop for Mexicans and especially for those inhabiting the Mesoamerican region for thousands of years, preceding Spanish colonization. Not only are the indigenous populations of this region responsible for the domestication of corn from its wild ancestor teosinte but share a cultural co-evolution alongside the biological evolution of the plant.

"It is time to return to our main character, to the mystery of its illegitimacy that still survives although it has reached a prompt endpoint, in order to remember the obvious yet frequently ignored: if the father of corn is unknown, its mother must be recovered, she is known and belongs to the indigenous cultures of that which is today known as Mexico. It is there, for millennia, where nature and knowledge have conspired to create corn, material sustenance of life and the subject of a large historical contradiction."

Arturo Warman
History of a Bastard: Corn and Capitalism, 1988

In the recent *4th festival of the Milpa* held in 2010 in the remote highlands of Oaxaca, Zapotec community leader Rodrigo Santiago Hernandez opened the ceremonies by emphasizing the long-lasting bond the people of Mexico have with corn "We plant corn for the well-being of the communities, if we don't cultivate corn, we have no life. It is central to our existence. We are the people of corn" (Ryan, 2010). The connection between Mexico and corn runs deep not only culturally, but as emphasized above by Arturo Warman, also biologically. Southern Mexico is recognized as the birthplace of corn, where the domestication of the species initially took place and consequently the greatest biological diversity of corn is also found there (Brush and Perales, 2007). A multitude of uses for corn are found alongside thousands of varieties and landraces, and utilitarian nutrition interplays with spiritual fulfillment through the Aztec god Centeotl, the Mayan Tonsured Maize God and others (González, 2010; Wertz, 2005).

Warman likens corn to a bastard in his exhaustive study of the history of the crop. Amongst other metaphorical parallels he draws through its illegitimacy, the above excerpt refers to the mystery surrounding the species' taxonomic antecessors. Warman's book was published in 1988 and since then further light has been shed on the unknown father(s) of this species. But the large contradictory history of the plant has taken a further turn at the dawn of the 21st century, one not foreseen at the time, but with immense consequences beholden, especially for Mexico and its indigenous cultures who have provided maternal stewardship for millennia. The introduction of transgenesis into the plant's genome is not only a further bastardization of corn but also an urgent call to trace the opaque lineages of newer generations of the crop.

The first commercialization of transgenic varieties of the crop by the agricultural biotechnology industry was in the mid 90s. These transgenic varieties were developed by crude genetic manipulations introducing foreign DNA (outside of normal reproductive processes) to express either bacterial toxins or to exhibit resistance to wide-spectrum herbicides (Pretty, 2001). Shortly after commercialization a chance discovery by our lab led to the first documentation of transgenic DNA being introgressed into native land races (Quist and Chapela, 2001). Significantly this introgression was found in the birthplace of corn, in the highlands of Oaxaca. The controversial paper,

which was published in the pages of the prestigious journal, *Nature,* set the stage for a scientific and technical battle for years to come.

A Question of Technics Tied to Politics

After the initial publication of the paper many critiques were leveled against it, both in the later issues of the journal as well as in the press and various public forums (Dalton, 2003; Hodgson, 2002; Kaplinsky et al., 2002). Although the critiques, verging on ad hominem attacks, were claiming both bias and lack of scientific rigor[1] none disputed the papers main finding, that transgenic, artificial DNA fragments were found within the nuclei of native landrace corn seeds in Oaxaca. As put by the lead author of that study:

> "The attention drawn to our research on transgene contamination of Mexican maize was important for at least two reasons. First, it brought attention to the need for careful evaluation of GMOs, especially in centers of crop origin and diversity. Second, the resulting controversy has led many to question whether the scientific enterprise, now dominated by economically influenced scientific agendas, has veered off the path of objectivity, free inquiry, and constructive discourse. Truly, the social and political complexities of "doing" science are beginning to rival the complexities of the natural world. Deliberate and overt corrective actions will be necessary to keep intact the guiding principles of interest free, self-critical scientific research, especially in the area of technology development and risk assessment. This is particularly true today, in the area of biotechnology, where invasive economic interests dominate the scientific agenda of what questions get asked, and what research gets funded. Without protections form such interests, whole modes of inquiry, some perhaps with the potential of new, unforeseen innovation, will be lost. The changes required ahead will require a commitment to the ideals of free inquiry not only by (sic) individual researchers, but the scientific community as a whole"(Quist, 2004)[2]

[1] The main technical issue the critics of the paper had was concerning the use of inverse PCR, a technique to determine where in a genome a particular sequence of DNA has been inserted. While there were some errors regarding the use of inverse PCR, and despite the further qualification of their statements by Quist and Chapela, this was used to discredit the papers main finding of introgression.

[2] Quist makes the case for freedom as a function of objectivity and sources of funding, which stands in contrast to the notion of freedom presented in this dissertation which is associated with embracing a political position.

A challenge on the presence of transgenic DNA in Oaxacan landraces would appear 4 years later. This paper, published by Ortiz et al. (2005) in the Proceedings of the National Academy of Sciences, gives us the opportunity to examine the way in which technical decisions and methodologies influence scientific findings while at the same time being wrapped up in a web of social and political considerations. Setting out to corroborate, or disprove, the results from the 2001 Quist and Chapela paper, the team of researchers went to the same zone in the Sierra Juarez with the goal of conducting a wider and more rigorous sampling effort. Despite having shown transgenic introgression, the original paper did not provide any clues as to the extent of the phenomenon since it only had a sample size of six. In order to improve on that insufficient sampling, the researchers of the new study collected 870 cobs from 124 fields.

2003			2004		
Total number of fields	Total number of maternal plants sampled	Total number of seeds analyzed	Total number of fields	Total number of maternal plants sampled	Total number of seeds analyzed
43	164	50,126	81	706	103,620[b]

Table 1. Sample sizes of fields, maternal plants (i.e., cobs), and kernels in 2003 and 2004 [Adapted from Table 1 (p. 12341), Ortiz-García S, Ezcurra E, Schoel B, Acevedo F, Soberón J & Snow AA (2005) *PNAS* **102**, 12338-12343. Copyright 2005 National Academy of Sciences, U.S.A)

Figure 1.1. Sampling data set (Ortiz-García et al., 2005)

The wide-scale field sampling allowed the researchers to claim to have a sample size of 153,746 but a careful reading of the article once again proves that the devil is always found in the details “Our strategy for determining frequencies of seeds with at least one transgenic parent was to analyze samples of pooled seeds to be able to detect rare, transgenic seeds at a reasonable cost”. Pooled samples allow investigators to cut down on costs and in this case more importantly on the amount of time necessary to process samples (Sham et al., 2002). But this occurs in place of a more precise and higher resolution of detection, analyzing each seed individually. The prioritization of cost and time over rigor and precision reminds us of market-based decisions of profit and liability. This is no coincidence as a commercial company in the business of detecting transgenes conducted the molecular analysis for the research in question also landing an employee as co-author of the paper. The tension and contradiction between scientific rigor and commercial profitability was so great in fact that it resulted in discrepancies between claims as to the limits of detection found in the publication and those in the documents of the actual commercial company conducting the analysis. The authors of the paper state that the companies used to conduct the DNA analysis

“Genetic ID and GeneScan routinely quantify 0.01% transgenic material, i.e., one transgenic seed in a sample of 10,000 with a degree of accuracy that is close to 100%...”(Ortiz-García *et al.*, 2005) According to Genetic ID’s own document on limits of detection; “In fact, the absence of a PCR signal in a single sample cannot be used as evidence that the DNA preparation did not contain GM material at the 0.01% level, since a certain number of DNA samples will fail to contain GM targets, even though such targets are present at an average frequency of 1 in 10,000 (0.01%) in the DNA preparation” (Genetic-ID, 2005). A clear contradiction between what the authors of the paper say the company can do and what the company itself says it can do.

Above and beyond sampling, pooling and amplification questions there also exists an even more serious oversight, almost too egregious to blame on a technical shortcoming. The gaze of the scientist is facilitated by instrumentation but there is no instrument that can correct for the mere refusal to see. “A negative score indicates that no band was detected by visual inspection, including faint bands that might indicate a transgene frequency of 0.005%. In all cases, and for both PCR methods, replicates from the same composite sample gave the same result, which was negative for both the 35S and NOS sequences.”(Ortiz-García *et al.*, 2005) Despite this declaration, simple visual inspection of the images presented in their paper tell a different story. The image below, prepared by Ignacio Chapela elaborates on this point.

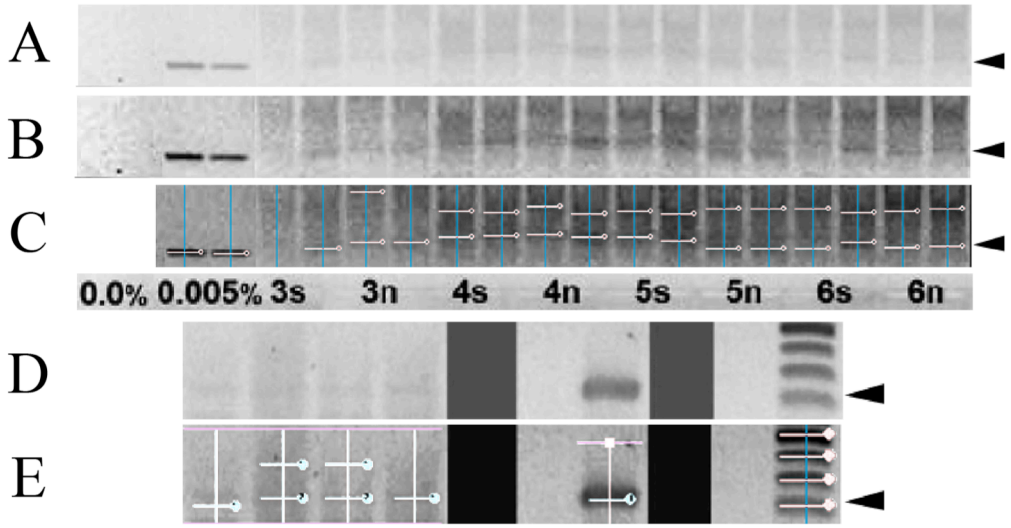


Figure 1.
Comparison of evidence used to suggest absence (A-C; Ortiz-García *et al*., 2005) or presence (D, E; Quist & Chapela, 2001) of transgenic DNA during the years 1999-2003 in local landraces of corn obtained from nearby locations in Oaxaca, Mexico. This comparison shows how very similar results have been interpreted to represent diametrally-opposed conclusions concerning the fundamental question of presence/absence of transgenic contamination in a given place of interest. Needless to say, such contradictory interpretations lead to confusion by reasonable, intelligent observers.

Both studies were perfomed using very similar methods, viz. an end-point PCR reaction designed to produce a band on a gel if a transgenic DNA sequence was found (band present) in a sample. For each panel, arrow-heads on the right indicate the location along the vertical axis where bands would be expected if transgenic DNA was present in the sample in question. Ortiz-García *et al.* (2005) published the image presented (in cropped form) in panel A, declaring that this photograph did not have any bands, and thus deducing that, to the best of their efforts, they were unable to detect transgenic DNA in their corn samples. Their study followed Quist & Chapela's (2001), who looked for the same transgenic DNA sequence, in the same geographical region, only a couple of years prior. Ortiz-García *et al.*'s image reproduced here (panel A), in cropped form, directly from its published version in *Proceedings of the National Academy of Sciences*; while Quist & Chapela's image is reversed (black-to-white; Panel D), for comparison, from the original published in *Nature*. In addition, images were sent for blind analysis at Kodak Molecular Imaging Systems, New Haven, CT, where they were both independently- and blind-analyzed in 2006 using Kodak's Molecular Image Software v.4.0. Panel A: cropped assembly from original in Ortiz-García *et al.* (2005), with two positive controls on the left end of the panel; Panel B: same as A, with contrast digitally increased to make bands more visually evident; Panel C: Kodak blind analysis of image on panel B, showing lines where the software automatically detected bands; Panel D: cropped from original in Quist & Chapela, 2001; Panel E: Kodak blind analysis of image E, showing lines where the software automatically detected bands.

Figure 1.2. The Presence of Observable Bands (Chapela in Krimsky and Gruber, 2014)

The next chapter in the unfolding saga of the appearing and disappearing transgenes came in 2009 when Alma Piñeyro-Nelson and her colleagues published the third paper investigating transgenic introgression in the region (Piñeyro-Nelson et al., 2009). This paper set-out at its onset to tackle both the technical issue of sample size as well as the political issue of discrepancies between a scientific versus commercial method.

They organized their molecular analysis to include both an in house as well as double-blind detection in another laboratory and importantly a further blind analysis at the same commercial outfit that conducted the analysis for the Ortiz *et al.* research. The results were clear; there were transgenes in Oaxaca yet the commercial company was not detecting them. In a publication marking the decade since the beginning of the saga, Bonneuil et al. (2014) comment that "PCR-based molecular biology has a blindness to sampling issues; to the dynamic, bio-cultural, and extensive web-like functioning of genetic landscapes; and to the fluidity and diversity of maize genomes. Thus, a singular reliance on PCR-based approaches failed to order the messiness of Mexican maize gene landscapes into unequivocally robust tests. Yet, at the same time, this hegemonic culture – which linked laboratory norms to journal and peer-review normative standards and to media and political networks – erased from recognition and visibility some non-compliant forms of life and knowledge that may have helped understand gene flows, in all their exuberantly emergent, lively bio-social complexity."(Bonneuil et al., 2014)

It is clear that the appearance, disappearance and reappearance of transgenes in Oaxaca is not that at all but the result of different sampling procedures and the molecular methods employed. This is somewhat self-evident since evolutionary investigations have shown that DNA is extremely resilient to disintegration and preserves its fundamental composition, of course in the process accumulating many changes on the scales of evolutionary and geological time. This is the basis on which all modern phylogenetic evolutionary research is built upon. To come to a conclusion of absence then becomes much more a conclusion on the lack of resolving power.

Increasing Resolving Power

Today investigating the presence/absence of transgenes in ecosystems falls squarely in the realm of point *detection*. These techniques, if employed in a large-scale and consistent manner could possibly provide the basis for *monitoring*: the regular detection of presence or absence. But the ultimate goal for any ecosystem analysis must be *mapping*, the high-throughput analysis of thousands of geolocated samples (Fierer and Ladau, 2012). Our research has been and continues to be with the goal of developing the technical and social solutions capable of mapping.

Discounting methods of detection for individual plants, and instead concentrating on landscape level detection of DNA (i.e. in agricultural production zones such as fields or bioregions), one quickly realizes the social and scientific need to

improve resolving power as it is also clearly evidenced by the Oaxaca case. The current state of the art for making claims to the effect of presence and absence involves a process which iteratively samples and subsamples. For the sake of illustration and since the examples given previously as well as the research presented in this dissertation involves corn; let us take a corn field as our zone under investigation. Traditionally, what is done is that a certain number of cobs are chosen from the field at certain intervals, and this is where the first subsampling is conducted. The next step is to grind all the seeds from these cobs and take another subsample. DNA is extracted from this second subsampling and then another further subsample is removed for the purposes of detecting transgenes. There are at least 3 points where biological information is being lost due to the technical barriers of molecular analysis.

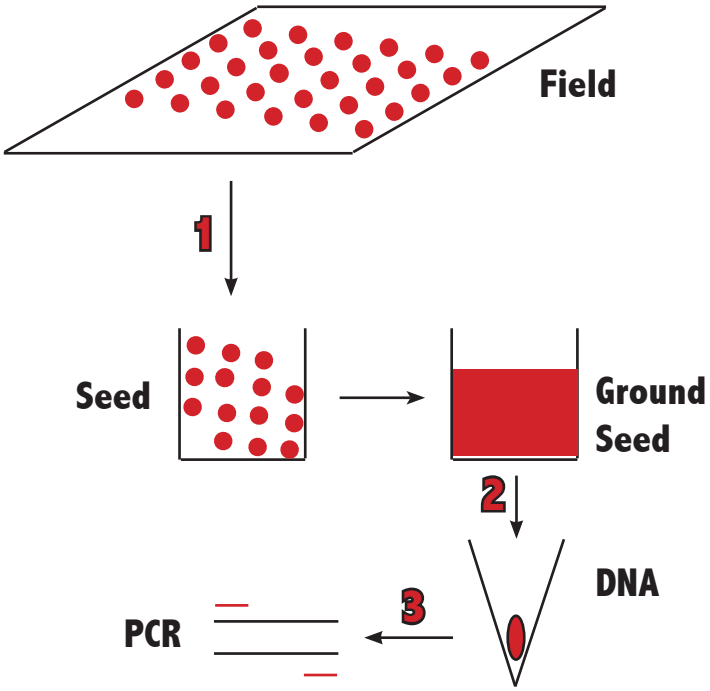


Figure 1.3. Three points where information is lost in standard field sampling protocols to detect transgenes.

Of course, any landscape level biological determination will involve a representative sample since it is practically impossible to conduct analysis on every nuclei of every cell and even every individual in the zone under investigation but the goal must be to minimize the subsampling while increasing individual point determinations and thus improving the resolution of biological information.

Three Domains of Mapping: Space, Time and Phylogeny

In the bourgeoning and dynamic field of Geographic Information Systems (GIS) two dimensions are most commonly visualized; space and time. The interaction of these two dimensions is what illustrates changes in landscapes. This is undoubtedly crucial and self-evident for any rigorous mapping of transgenes as well. The temporal dimension, the crux of any evolutionary statement, can be provided by not only stringent sampling of the same geographical area to establish yearly levels but also by querying sedimentation to go further back in time (Taylor et al., 2009) and establish a certain baseline of landscapes prior to the initial introduction of transgenes in 1987 in to the wild.

There is a further dimension that concerns those who are interested in tracking and mapping the movement of biological information, whether it is DNA or other material playing a role in heredity. This is the dimension of phylogeny. Despite widespread assumptions to the contrary, biological systems and species have dynamic and flexible boundaries. When it comes to hereditary information Alan Rayner describes this beautifully in his exceptional *Degrees of Freedom: Living in Dynamic Boundaries* " It is therefor at boundaries that all life's action occurs – the places where nature (genetic influences) and nurture (outside influences) combine and inextricably intertwine to generate the rich complexity of the living world. These boundaries can never be completely fixed, but instead define the ever-changing *contexts,* the local environments within and between which life processes are transacted across scales of organization ranging from microscopic to global. When it comes down to it, life forms are molded within dynamic contexts containing *wet genes,* and not assembled from dry building blocks" (Rayner, 1997). These flexible boundaries have immense evolutionary significance. It is well established that both the mitochondria and the chloroplast were once distinct microorganisms that began symbiotic relationships that evolved into mutualism(Margulis, 2008). Even further, horizontal gene transfer, facilitated by bacteria and viruses occurs more prevalently than acknowledged (Jain et al., 2002). "We know from a experiments in the laboratory and in controlled conditions, as well as from even fewer field-based observations, that horizontal gene transfer of transgenic DNA species occurs(Dröge et al., 1999). This means that DNA introduced into the environment—as part of an organism carrying it—will move not only within that species over time and through space (such as through cross-pollination), but also across the breadth of the phylogenetic landscape formed by the variety of life forms with which the original organism comes into contact. Gene transfer occurs during sexual reproduction within a species, and is therefore considered "natural." As the transfer involves organisms which are less likely to exchange DNA without the increased-promiscuity functions of transgenic DNA species, the term "horizontal" is used." (Chapela in Krimsky and Gruber, 2014)This and other biological processes facilitate the interchange of information across phylogeny (Syvanen, 1994).

This dimension takes on an even more central role when one considers the transgenic constructs themselves and the processes of their creation. The movement of chimeric DNA sequences, often transgenes will include DNA from 6 or more distinct species and individuals, into living organisms is only possible by manipulating natural processes of horizontal gene transfer, such as the transference of DNA from one species to the other by the pathogenic *Agrobacterium tumefaciens* (Ishida et al., 1996). The DNA insertions in transgenic species continue to carry sequences which are known to facilitate their uptake and movement by microbes (Cummins et al., 2000). This presents the potential of higher rates of movement in the phylogenetic dimension in comparison to relatively more stable DNA in species that are products of natural evolution and not laboratory manipulation.

Detection and mapping of Airborne Particles by A Distributed Network of Partisans

The improvement of the landscape level resolution of detecting DNA-based biological information is necessarily tied to the subject undertaking the investigation. Just as the periodic sampling of a small number cobs and/or other plant material are insufficient for landscape level claims, the small number of researchers housed in universities and/or other institutions are also insufficient. We are faced with a challenge to improve both the biological object of our detections as well as shift the subject undertaking these investigations. Once again we are faced with the intertwining of the social and technical.

If our goal is high-throughput sampling for the detection of the ebbs, flows and fluxes of the movement of transgenic DNA in plants, the self evident object of analysis is the primary vector of reproductive information exchange found in flowering plants; pollen grains. The airborne nature of pollen grains also enables easily sampling and storing a large number of discrete particles over a large geographical zone. Pollen grains sampled from a flowering field of plants provide a previously unimaginable amount of biological data packaged into discrete, resilient microscopic units.

In contrast to the current field-based point sampling, airborne sampling and mapping of DNA from pollen can also provide a window into the phylogenetic movement of transgenic DNA by not only querying the particles for presence/absence of such transgenes but also by well-established markers for species identification.

As stated previously, an integral goal is to undermine the hegemony of biological research conducted by professionals housed in research institutions who obscure their ideological role in society by claiming neutrality, objectivity and benevolence. The facilitation of a distributed network of partisans, armed with devices capable of surpassing the subpar resolution of “objective” researchers can start asking the questions obscured by prevalent research paradigms. Some of these questions might be: are there correlations between social class and the movement of transgenes? How is human, animal and ecosystem health affected by transgene concentration? How is

species diversity affected by transgenes? What are the evolutionary implications of the movement of transgenes through phylogeny? These are only a few of the cursory questions that might be proposed but the most interesting ones are surely to emerge from within a distributed network of partisans.

For us, a “distributed network” implies a horizontally organized network of investigators who collaborate and organize with each other through free association. This collaboration not only results in joint research projects but also validation/refutation of results from one node in the network by the other. Partisanship implies being clear and transparent about political and intellectual allegiances and committed to goals antagonistic and counter to the social political system, i.e. partisans in a social struggle to overcome the world we live in. The network is made up of conglomerations of coherent political entities capable of taking action as it sees fit via horizontally-organized decision making structures.

The bulk of this dissertation is focused on the technical: the molecular biology central to the creation of such devices facilitating the autonomous mapping of airborne microbes, in this case specifically airborne pollen, with the goal of mapping the movement of transgenic DNA. Through the following pages the reader will be presented with a brief review of the history of the devices and biochemical reactions of the same vain, three articles either published or submitted as manuscripts for publication which outline the principles of our method and a concluding chapter tracing our work so far with beekeepers and honey distributors as well as future directions.

Chapter 2:
The Muddy Terrain of Detecting Microbial Life

Before presenting the methods we have developed in our laboratory to accomplish the goals laid out in the introduction, it is useful to review the state-of-the-art in microbial DNA detection, and their various strengths and shortcomings. A review of the field, beyond its techno-scientific features, and including its sociopolitical characteristics also enables us, and our political project, to take position at a strategic place and time.

Three intertwined fields: Biosafety, Biosecurity and Biodefense

The technical development of detecting microbes in the air via their DNA takes place within the confines of three distinct yet intrinsically intertwined fields; *biosafety, biosecurity* and *biodefense*. Biosafety can be traced to the origins of the liberal environmental movement, commonly marked by the publication of Rachel Carson's indictment of pesticides in *Silent Spring* in 1962 and the establishment of the EPA in 1970 (Carson, 2002; Marco et al., 1986).

As defined by the Cartagena Protocol on Biosafety "Biosafety is a term used to describe efforts to reduce and eliminate the potential risks resulting from biotechnology and its products. For the purposes of the Biosafety Protocol, this is based on the precautionary approach, whereby the lack of full scientific certainty should not be used as an excuse to postpone action when there is a threat of serious or irreversible damage" (Secretariat of the Convention of Biological Diversity, 2000). On the other hand, the arena of Biosecurity sits squarely within the domain of warfare, beginning, in its modern incarnation, with the confrontation between technological superpowers during the First and Second World Wars. There is a resonance between Biosafety and Biosecurity in that they both emphasize the precautionary approach, preemptively seeking to "ensure" the safety/security on one side of a conflict, without implying directly the use of proactive aggression. As a separate but closely related concept, Biodefense comprises more proactive use of biological agents as warfare agents. Whatever the conceptual framing for these fields, they all share the urgent need to provide methods and techniques, instruments and technologies for the quantification—or at least *detection*—of microscopic (microbial) life-forms such as bacterial cells, fungal spores, pollen grains, virus particles, and so on.

The US biowarfare program matured in the 20th century as the war and scientific establishments were brought together under the same roof. The most direct effort towards this took place during the presidency of Franklin Delano Roosevelt, who protected and promoted the work in the lab, the field and the policy arena of the charismatic and exceedingly influential Vannevar Bush (Bush, 1945, Zachary, 1997).

Among many institutions and collaborations, which eventually emerged from this process, the JASON project became the primary, and elite, think tank of the intelligence community looking into techno-scientific questions and their solutions. Born during the Manhattan Project, matured during the Vietnam war and called upon in the post-9/11 War on Terror, the JASONs are a team of mostly academic physicists (more recently also biologists) who advise the Pentagon on a diverse range of topics. Charles Schwartz, a physicist at UC Berkeley provided one of the first public exposes of this secretive group in his 1972 pamphlet titled *Science Against the People:* "This special scientific study group was assembled under the auspices of the Jason Division of IDA; the group of 47 scientists represented "the cream of the scholarly community in technical fields"..."a group of America's most distinguished scientists, men who had helped the Government produce many of its most advanced technical weapons systems since the end of the Second World War, men who were not identified with the vocal academic criticism of the Administration's Vietnam policy." This Jason study group met during the summer of 1966, starting off with a series of briefings by high officials from the Pentagon, the Central Intelligence Agency, the State Department and the White House. They were given access to secret materials." (Schwartz, 1972)

Ann Finkbeiner, in her history of the JASON project describes their newfound relation to biology:

> "The biologists' war is, of course, biowarfare and bioterrorism, the weapons being disease-causing microbes. Biological warfare by one country against another has limitations – clouds of microbes are inefficient killers, hard to target precisely, and hard to spread effectively – that make it ineffective as a national strategy. Biological terrorism by and against individuals – the same clouds released in a subway or on an airliner—is unhampered by those limitations and therefore much more effective. In 1999 Jason did a study for DARPA called "Civilian Biodefense" that predicted how the latest in genetic engineering might not only change the character of microbes used as weapons but also move the battlefield from the military to the civilian. The study also said that though sensors of biological weapons were available, neither the federal government nor the health care profession was prepared to turn the sensors into an interconnected system that could detect and respond to an attack. It suggested that the best detection/response system might be the existing public health system and it recommended additions to the system. "It was a very good report," said Frank Fernandez, who was DARPA's director at the time and had commissioned the report. It was briefed up the line and other agencies picked up some of the surveillance techniques. The techniques are being pursued."(Finkbeiner, 2006)

In February 2003, JASON prepared a report titled “Biodetection Architectures” on the potential of detecting microbes within the rubric of biosafety. They concluded as follows:

> “JASON considered the essential components and operation of an effective strategy for homeland biodefense based on technologies that are currently available or likely to become available within the next five years. It is not realistic to undertake a nationwide, blanket deployment of biosensors. This might be done for the detection of airborne anthrax, albeit at substantial cost. However, there are many possible bioterrorism agents and many possible ways in which they can be delivered. Instead, biosensors should be deployed in a focused manner as one component of a broader biodetection architecture that also includes information derived from intelligence gathering and medical surveillance. This information should be analyzed by a team of local experts who are familiar with local vulnerabilities, high-value targets, and environmental conditions. The local analysis team also should be responsible for directing an appropriate response in the event of a bioterrorism attack. They will be guided by a pre-established “playbook” that recommends particular responses for a particular set of circumstances, which will have been practiced and refined through staged exercises.” (JASON, 2003)

Biosecurity, biodefense and biosafety may appear to be different and even oppositional. It would be easy to assign specific political epithets to each: biodefense as a “conservative” position set against the “liberal” biosafety, with biosecurity positioned somewhere in between. For the biosafety discourse, microbial agents are agents of undesirable outside antagonists (corporations, specific nation-states, etc.), whereas for the biosecurity discourse the antagonists are foreigners and their political interests. All three fields, however share in common a view of microbial life-forms as sources of concern or outright enemies, as alien invaders to be avoided. With the increase of non-state adversaries to the dominant powers of the world, there has been increased talk of non-conventional weapons of biological warfare. Regardless of how much of this is based in reality and how much of it hype, this climate of biofear has brought considerable funding and research into detection machines (Reppert, 2005).

Biodefense, biosafety and biosecurity all purport to enable biological control via biological detection. Resting upon the paradigm of nation-states and borders, this control claims to enable confinement of certain undesirable microbes to certain geographical zones. Microbial life resists containment, mostly due to its small size, large scale and reproductive success. But even further when we turn our attention to the DNA (and other hereditary material) found within these microbes the control becomes even

more improbable due to the fluid and constant exchange of such material between microbes of different species and higher organisms as well (Jain et al., 2002).

Detection and Surveillance

In recent years the two fields of Biosafety and Biosecurity have merged under the guise of global emergencies caused by pandemics. Considered a national security issue, governments have poured significant resources into companies, research institutions and other outfits promising to detect the origin and spread of pandemics. This effort is being led in multiple avenues ranging from monitoring social media to on the ground biological detection and surveillance.

Perhaps the most expansive of these efforts is one conducted by the non-profits Global Viral and Metabiota(Global Viral, 2015, Metabiota, 2015). Founded and run by Stanford Professor Nathan Wolfe, Metabiota defines one of its goals as follows:

> "Understanding the origin and progression of emerging, re-emerging, and novel infectious diseases is critically important to preventing epidemic and pandemic outbreaks. Whether naturally occurring or intentionally introduced, such diseases pose a massive risk to global health and require active vigilance for signs of outbreak, rapid recognition, and accurate diagnosis of the microbial cause. "(Metabiota, 2015)

Their approach provides a good example of the prevalent research paradigm into the detection of microbial life. It consists of collecting samples form infected individuals and bringing these samples to centralized laboratories for DNA sequencing and analysis. From the point of collection and analysis such centralization limits the amount, speed and kind of data generated; it also establishes a very high minimum cost for even the possibility of *mapping*, given the expense involved in collecting and processing of samples by highly-paid, highly-trained experts (post-docs, graduate students, interns, etc.). But even further, the medical surveillance conducted by or for governments for the detection of global pandemics has the potential for infringing on enlightenment values such as privacy (Korn, 2001). This approach also continues to propagate the notion of the professional researcher as the purveyor of biological information, further complicated in the case of Global Viral and Metabiota, as western researchers facilitate the collection and analysis of samples collected mainly from Africans, and in doing so continue a colonial legacy of racist relationships. The techniques used to conduct the research are as important as who is conducting the research and along what lines of social organization.

Social Organization

In her treatment of the JASON Project's 2003 Biodetection Architectures Report, Finkbeiner explains that "It began by saying that in five biodefense studies done over the previous five years, Jasons had become increasingly frustrated with what they called the "near-pervasive focus on biodetection gadgetry." What the country still didn't have, they said, was a way to turn the gadgets into a system that could be set up at that moment in the real world; they suggested ways to create a system that would be "nimble," would be capable of being installed quickly, and would change as the threat changed" (Finkbeiner, 2006). The Jasons realized that, technics cannot act alone as mere machines in a social void.

Mobile yet Centralized

Rapid detection of microbial life has developed under the priorities determined by biosecurity in the face of a biological attack or halting the spread of infectious diseases. Biosecurity, like all that falls under the rubric of national security, is fundamentally also about information control, and this places its biodetection under tight supervision.

The technical solutions are therefore constrained by the political considerations. And the political considerations that centralize microbial detection prevent novel technics from being developed. The state-of-the art in biosecurity detection of microbial DNA involves rapid dispatching of mobile labs to a site of interest to conduct on-location analysis or more commonly the shipment of samples to a centralized facility for analysis (Battelle, 2015, Petro et al., 2003)). When this is the detection schema, naturally the development of autonomous technics becomes a mute point and thus, these labs and their personal rely heavily on well-established yet costly and professionalized methods such the Polymerase Chain Reaction.

When it comes to high-throughput methods geared at processing a large number of samples in a small amount of time the proposals are also just as centralized. Almost all high-throughput solutions are based upon liquid handling robotics ranging from processing 96 or 384-well plates, to microfluidic reaction vessels or even extreme solutions such as an array tape (Sundberg, 2000, Douglas Scientific, 2015). These instruments are not only extremely expensive but also require dedicated facilities, trained personal and continuous maintenance that place them beyond the reach of any wide-scale deployment in the world.

Distributed yet Vertical

Although biosecurity driven microbial DNA detection has not been considered for alternative methods of organizing investigations, a distributed organization of

research has been proposed in the sciences. Bioinformatics, protein analysis and even biomedical surveillance have brought forward ways of organizing their research which have suggested distributed models. These projects fall under what is classically referred to as *participatory citizen science*.

The crucial aspect common to these distributed models is that they embody a vertical structure with unidirectional flows of sample and decisions (Figure 4.1a). Although certain aspects of the work is distributed, this distribution is more on the level of acquiring samples or computing power in the service of the primary and central research team and their research goals. Citizens in the distributed network do not have agency to determine research questions and often access to the data that they and their peers generate. The actual analysis of samples is usually conducted in centralized facilities. Nonetheless the impetus to participate by citizens is great and arises from genuine curiosity, unfortunately stifled by the top down distribution.

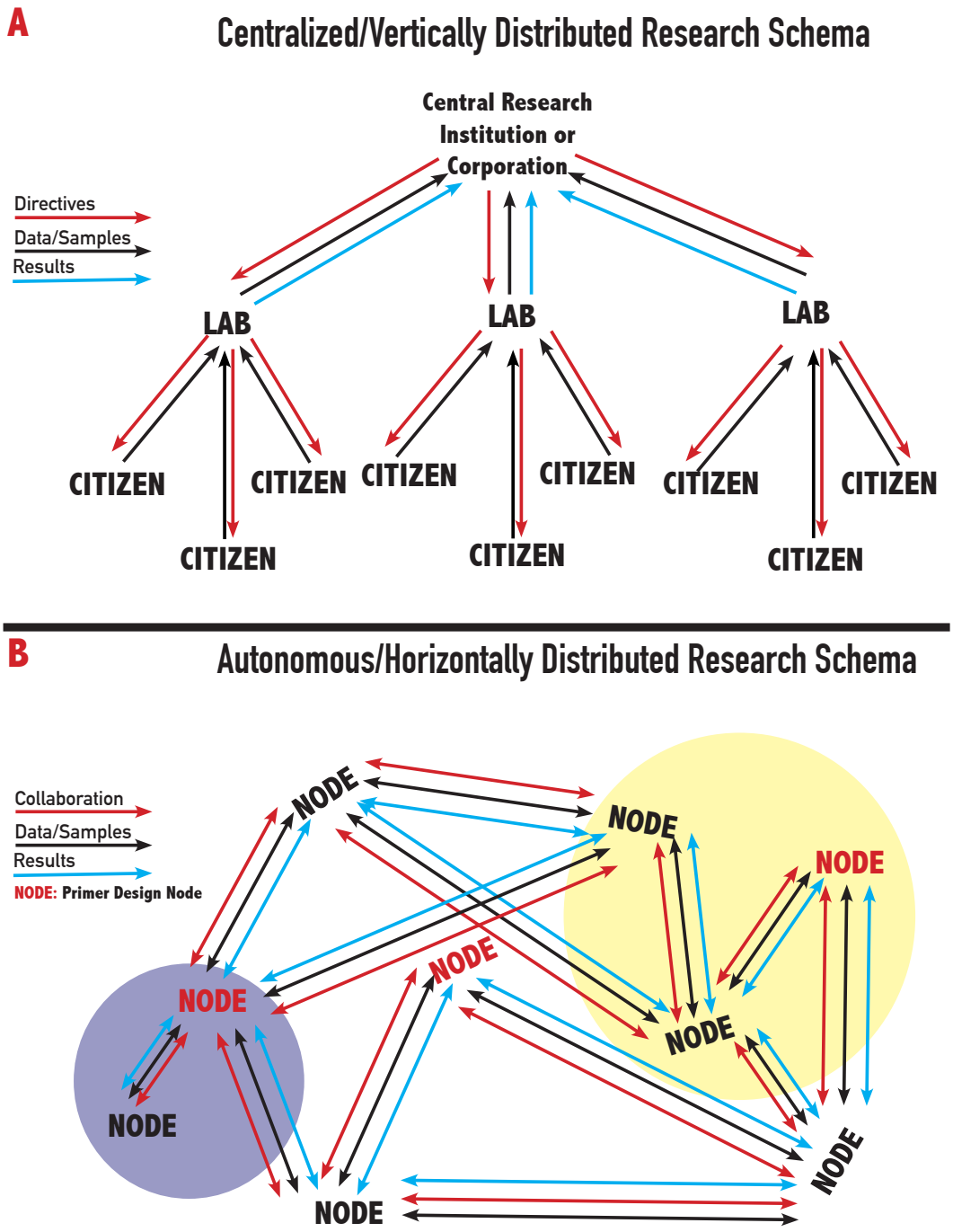


Figure 2.1 ***(A)*** *Representation of a centralized/vertically distributed research schema, contrasted to* ***(B)*** *an autonomous/horizontally distributed research schema. In the Autonomous/Horizontally Distributed model there is a further dimension of spheres of collaboration, where different nodes converge to work together.*

Mobile, Autonomous, Distributed and Horizontal Detection of Microbial DNA

We are proposing to invert the current models of detecting microbial DNA, both with technical innovation as well as a different social organization of those conducting the detection and directing the research agenda. Our technical goals are driven by our social priorities. With this vision, horizontally organized nodes are in possession of devices capable of detecting microbial DNA autonomously (without having to send samples to a centralized facility). In its ultimate iteration (presented in the concluding chapter) these devices must be able to sample thousands of data points and conduct analysis on them without the need to utilize expensive robotics.

The social organization of this network is horizontal (fig 4.1b) and enables its participants to have agency in deciding which research questions to prioritize. Nodes relate to each other under principles of free association and collaborate according to their own interest in pursuing a particular project or not. This organization also allows for the coordination in validating or disqualifying results from one node by the other, identifying gaps in research programs and remedying them.

Amplifying DNA: Rapid Thermal Cycling versus Isothermal Amplification

If technics are intrinsically tied to ideologies it is essential to examine some of the current methods of detecting DNA from microbial life and identify that which is most conducive to ours. Certain methods of investigating the presence and absence of microbial life will be omitted here, such as ELISA kits using immunological methods, microscopic observations or air sampling devices that use fluorescence or light scattering to identify microbes. These techniques are omitted due to our focus on microbes possessing transgenic DNA. This is an important distinction because, evidently, microbes (pollen or otherwise) carrying transgenic constructs are indistinguishable morphologically (Wrubel et al., 1992). More relevantly, immunological kits, assume that the DNA sequence in question is being translated and transcribed to produce its associated proteins. This might be relevant for some detection efforts but any detection of DNA sequences cannot make such an assumption since the truncation, rearrangement and replacement of transgenic DNA have been amply demonstrated (Bhat and Srinivasan, 2002; Nakano et al., 2005). Therefore any true detection of microbial DNA must focus on the DNA molecule itself and not its assumed phenotype consequences such as morphology or proteins.

For the purposes of this undertaking we will contrast two ways of detecting DNA, which differ fundamentally in how they create template strands for the iterative and exponential amplification of the sequence in question. On one hand we have the conventional method based on the rapid cycling through temperatures almost 30°C apart and on the other hand an isothermal method, ingeniously obliterating the need for rapid cycling.

Thermally Cycled DNA Amplification: *PCR*

Currently, by far the dominant method of detecting DNA is amplification through the Polymerase Chain Reaction (PCR). Developed in the 1980s by researchers in the Cetus Corporation (Rabinow, 1996), it has dominated molecular analysis for decades. While some improvements have been done to increase the speed, precision and reporting of results, the essential elements of PCR remain the same to this day. It is an iterative, exponential process of amplifying a target DNA with the assistance of a thermostable polymerizing enzyme (Saiki et al., 1988). The key to PCR is the repeated denaturation of double stranded DNA, annealing of the guiding primer pair, and the elongation of single strands by a DNA polymerase. These three distinct steps take place at three distinct temperatures therefore requiring the rapid cycling of the reaction vessel between them (Mullis et al., 1994). The state-of-the-art for cycling through these temperatures is to do so with a device aptly named a thermocycler, which uses the Peltier effect to accurately and rapidly modulating temperature by electricity (Atwood et al., 1995).

The requirement of a relatively expensive device capable of cycling through temperatures determines who can use it and where it can be used. This is one of the reasons why DNA detection cannot take place, under current conditions and using the PCR reaction, outside of commercial, academic or non-profit laboratories at worst and in mobile, temporary labs setup by experts in the field at best.

Simple as the PCR may appear to the observer or practitioner in a country where instrumentation costing thousands of US dollars is not really limiting, where electricity is abundant and reliably regulated, where distribution of instrumentation, repair and maintenance capacity is a given, the PCR method is practically unattainable in the context of remote field situations or impoverished regions.

Isothermal DNA Amplification: *LAMP*

In the past decade a number of different methods for the isothermal amplification of nucleic acids have been developed. Some examples include rolling circle amplification (RCA) (Demidov, 2002), which uses a circular template to generate concatemers, strand displacement amplification (SDA) (Walker et al., 1992), helicase-dependent amplification (HDA) (Vincent et al., 2004) or nucleic acid sequence-based amplification (NASBA) (Deiman et al., 2002), which is specific to amplifying RNA and loop-mediated amplification (LAMP) (Notomi et al., 2000a).

Of the isothermal methods which act on DNA rather than RNA, LAMP stands out in contrast to the others and has presented the most promise and widespread adaptation in a variety of fields. One of the most obvious advantages is that the polymerase employed has been demonstrated to be exceptionally resilient to environmental contaminants (Francois et al., 2011) and therefore perfect for field-based

detection. Additionally, the at least 4 oligonucleotides which are specific to 6 different priming sites increases the specificity of LAMP. The exceptional aspect of LAMP is that it is able to produce an immense number of amplifications (approximately one billion in an hour (Asiello and Baeumner, 2011). This allows for a host of techniques to be employed in order to visualize the end product of the reaction that range from various fluorescent to electrochemical methods to even the simple observation of precipitated byproducts of the reaction.

Developed by Japanese researchers at the turn of the century and quickly incorporated into the biotechnology company Eiken, LAMP has attracted the interests of entities ranging from the Bill and Melinda Gates Foundation (Hsiang et al., 2014) to detect malaria in Africa to the Sydney Water Company to test water samples (Mikosza, 2011). The majority of the work on field-based LAMP is being done for the detection of infectious diseases in the global south and point-of-care devices for use in hospitals.

LAMP differs form PCR in its use of an enzyme isolated from *Bacillus stearothermophilis, which* has a 5' to 3' strand displacement activity in addition to its polymerase activity (Swaminathan and Wilkosz, 1998). This characteristic of *Bst* allows LAMP to bypass the denaturation step integral to PCR. Furthermore, the optimum temperature for the polymerase activity of LAMP is within close range of primer annealing temperatures allowing for isothermal amplification.

The use of 4 or 6 primers in LAMP allows for greater specificity due to the greater amount of annealing sites required to produce a positive reaction. It is important to note that this also implies that more care must be taken when designing the primers in order to prevent possible mispriming. The second feature of LAMP is that its mechanism of extending dumbbell shaped chains (Figure 2.2) allows a great number of amplification products to be synthesized in a relatively short amount of time in comparison to PCR. This efficiency of producing amplicons can be harnessed in conjunction with fluorophores to report their presence or absence in the form of fluorescence, so strong in fact that it is even observable by the naked eye. This is one of the clearest advantages of LAMP and makes the use of toxic intercalating DNA stains such ethidium bromide or costly fluorescence detection instruments unnecessary.

Having roughly sketched out the field we find ourselves operating in, let's now turn to the specific technical achievements we have accomplished in the laboratory. What follows is a collection of 3 articles that lay out some of the initial steps as we develop the capacity to detect microbial DNA in the field.

Figure 2.2 *Loop-Mediated Isothermal Amplification form Nagamine et al. (2002)*

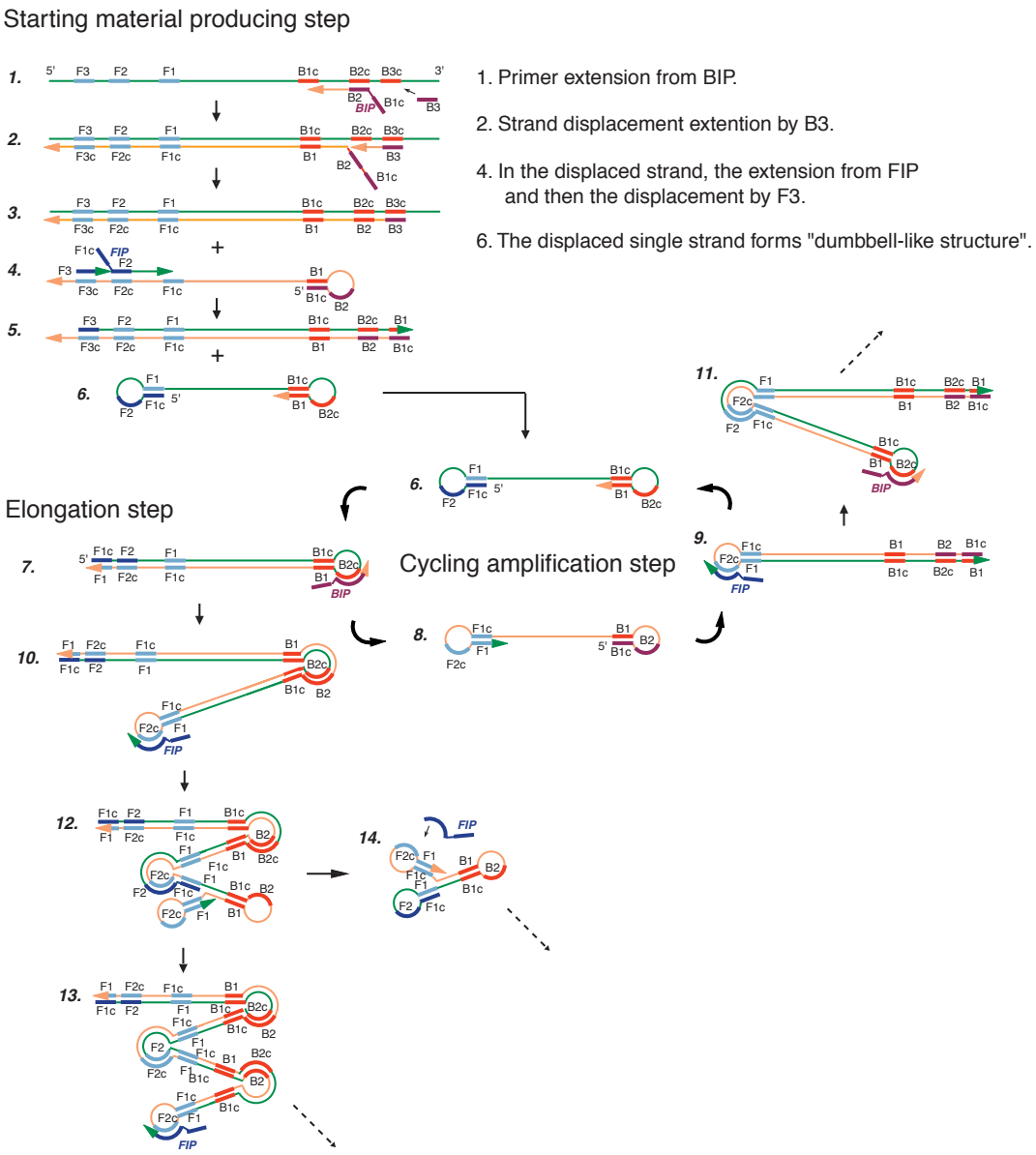


Fig. 3. Schematic representation of the LAMP mechanism using loop primers. (A) Steps in the original LAMP reaction. In the first step, in which starting material is produced, the dumbbell-like DNA form (structure 6) is generated. Then, in cycling amplification step, DNAs of this form are generated continuously. The elongation reactions are started from the sub-products (structures 10 and 11) of the cycling amplification step, generating various sizes of the products. F2c and B2c are hybridized by the inner primers.

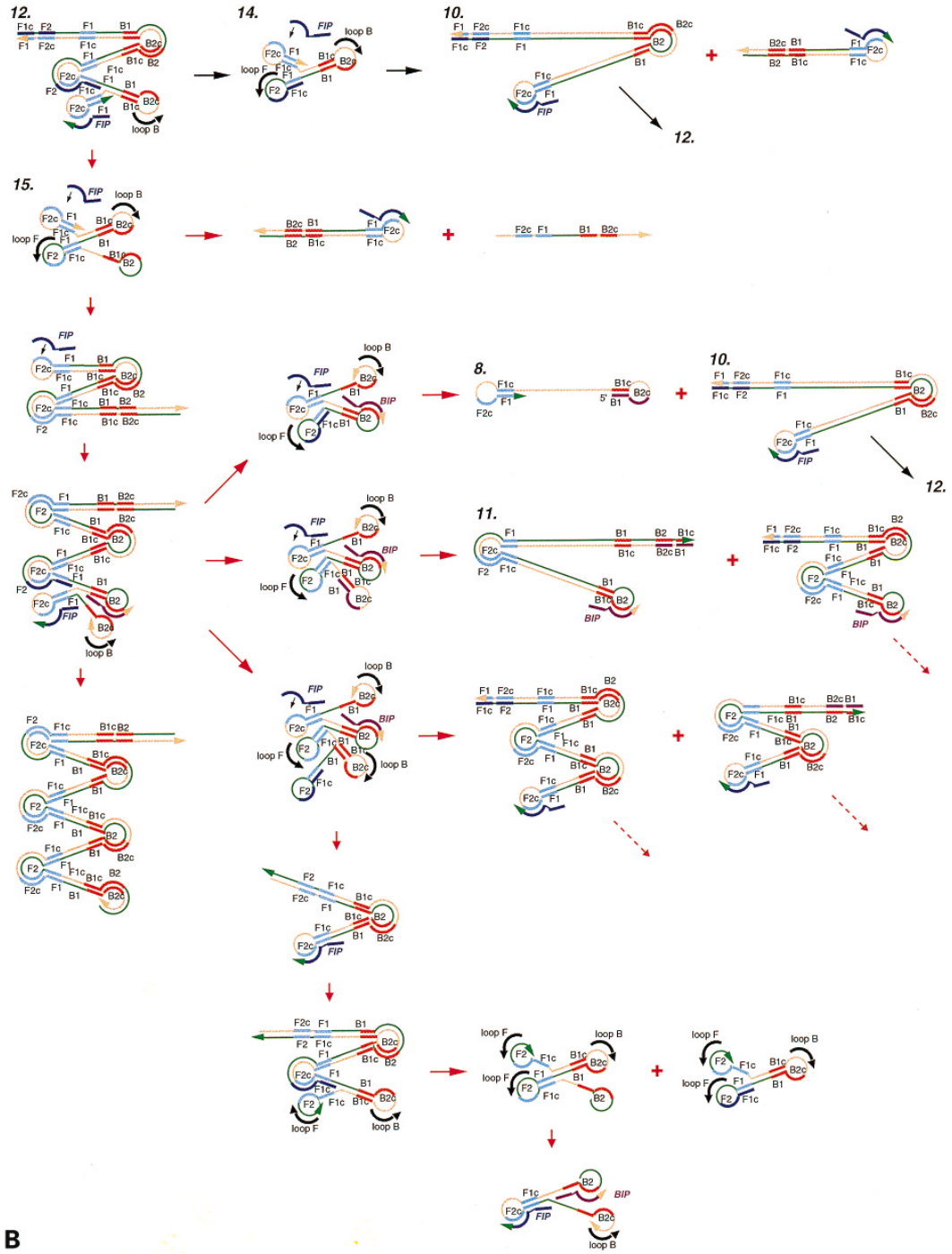


Fig. 3. *Continued*
(B) This figure shows the process that starts from structure 12. Black arrows show the original LAMP reaction pathway. Red arrows show the reaction derived from the loop primers. Red dotted arrows represent continuous reactions. The region between F1 and F2 (or B1c and B2c) is hybridized by the loop primer. The primers that hybridize the 3′ region of the products are not shown because they are irrelevant to the acceleration of amplification.

Chapter 3: *published in the Journal of Integrative Plant Biology (March 2014)*
Loop-Mediated Isothermal Amplification of Single Pollen Grains

By Ali Bektaş and Ignacio Chapela

ABSTRACT:

The Polymerase Chain Reaction (PCR) has been a reliable and fruitful method for many applications in ecology. Nevertheless, unavoidable technical and instrumental requirements of PCR have limited its widespread application in field situations. The recent development of isothermal DNA amplification methods provides an alternative to PCR which circumvents key limitations of PCR for direct amplification in the field. Being able to analyze DNA in the pollen cloud of an ecosystem would provide very useful ecological information, yet would require a field-enabled, high-throughput method for this potential to be realized. Here we demonstrate the applicability of the Loop-Mediated DNA Amplification method (LAMP), an isothermal DNA amplification technique, to be used in pollen analysis. We demonstrate that LAMP can provide a reliable method to identify species from the pollen cloud, and that it can amplify successfully with sensitivity down to single-pollen grains, thus opening the possibility of field-based, high-throughput analysis.

Introduction

DNA amplification is well established as a versatile approach to many ecological questions. Conventionally, amplification of DNA is achieved through the Polymerase Chain Reaction (PCR), typically using two primers to guide the synthesis, by a thermostable polymerase, of large numbers of copies of a DNA region of interest. In ecology, this allows for precise and sensitive generation of presence/absence data for the chosen DNA sequence in a wide range of applications (Smith and Osborn, 2009). Despite its proven utility PCR has limitations which make it less than ideal for ecological applications. To name a few, polymerases used in PCR are easily inhibited by common environmental “contaminants,” (Schrader et al., 2012; Wilson, 1997b) making it necessary to extract and purify samples; the reaction itself is performed through precise and sensitive temperature cycling, typically involving either trained labor (Harris, 1998) or relatively expensive laboratory thermocyclers; the result of the reaction in PCR is commonly visualized using toxic, carcinogenic chemicals such as ethidium bromide under UV radiation. These and other characteristics of PCR make for challenges when applying these methods closer to field situations.

Recently, DNA amplification methods have been developed that allow faster, more robust and field-compatible techniques (Asiello and Baeumner, 2011). In the last twelve years, a method called Loop-Mediated DNA Amplification (LAMP) (Notomi et al.,

2000b) has shown promise for ecological applications in field-based situations. The enzyme utilized in LAMP, DNA Polymerase I, Large Fragment from *Bacillus stearothermophilus (Bst)*, is a polymerase which can operate through nick-and-displacement of double-stranded DNA, eliminating the need to cycle temperatures such as in PCR where a denaturing (heating) step must be performed before the necessary annealing and extension steps, each at different lower temperatures (Mullis et al., 1986). This advantage is added to the generally more robust performance of *Bst* DNA Polymerase I, Large Fragment, which is not inhibited as easily as the PCR polymerases (Kaneko et al., 2007), and can be used inexpensively in conjunction with fluorescent (Curtis et al., 2009a; Mori et al., 2006; Tomita et al., 2008) or bioluminescent (Kiddle et al., 2012) end-point reporters to give a method which has very large promise in field-based ecological applications. Although many improvements to increase its sensitivity and performance have been achieved on the PCR method since its invention, the advantages listed above of the LAMP method are particularly suited for field and remote-location applications. LAMP has been proposed for field use in epidemiological studies of human (Abdul-Ghani et al., 2012), animal (Savan et al., 2005) and plant (Okuda et al., 2008) systems, as well as in more traditional laboratory-based studies. LAMP, however, remains relatively new in situations of direct ecological relevance and quantification.

Pollen clouds can be regarded as the air-based floristic footprint of spermatophytes in an ecosystem. Being able to provide fast, inexpensive, field-based analysis of pollen DNA can thus provide a powerful tool for molecular ecological studies. Furthermore, pollen provides an excellent unit for population biology analyses, since it carries only a small and discrete number of nuclei in the highly reduced and protected male gametophytic stage of higher plants. Because of the chemically resistant nature of pollen wall layers (Brooks and Shaw, 1978), DNA carried by individual grains in a pollen cloud represents a stable record of ecosystem structure that can be collected without special cryogenic or dehydrating equipment (ice, liquid nitrogen or silica gel). Accessing pollen from various moments in an ecosystem's history provides one of the most important sources of information about its floristic change over time, ranging from ecological to evolutionary scales (Matsuki et al., 2007; Mitchell, 2011). Last but not least, knowledge about pollen distribution in space and time has significant practical human implications in allergy studies (D'Amato et al., 2007).

We have developed a LAMP-based method allowing for the specific detection of DNA sequences in very small numbers of pollen grains, down to a single grain. Our goal has been to minimize the various technical requirements for a successful LAMP amplification in order to obtain a simple, inexpensive, rapid and field-compatible method, which could be expanded through miniaturization and automation of the processing and detection methods. Here we introduce the method and demonstrate its potential. We also discuss some of its limitations for field operability.

Results

DNA amplification using LAMP was successful down to the single-pollen grain level of resolution for all tested species. DNA amplification from single pollen grains (Fig. 3.1) is indistinguishable from amplification of extracted and purified DNA from the same species (Fig. 3.2). Unlike PCR, which typically yields a single dominant amplicon and gel band, LAMP produces a specific banding pattern for each primer set, a pattern that is repeated in each reaction irrespective of whether the template DNA was introduced in purified or pollen-bound form. This level of very high sensitivity and robustness is a consistent result across all 9 plant species we have tested, namely *Alnus glutinosa, Zea mays, Cedrus deodara, Salix sp., Juglans* hindsii*, Corylus* cornuta, beyond the three examples used here.

Increasing the number of pollen grains up to at least 50 for each reaction did not affect the results qualitatively, giving a reliable amplification without the need to extract or purify DNA from its environmental context (Figure 3.3). No inhibition of the reaction by the constituents of pollen has yet been observed in our experience (with positive reactions occurring in the presence of millions of grains), with the ultimate limitation to the number of grains present being the reaction volume.

Using multiple pollen grains per reaction, but still without extraction or purification of DNA we showed that LAMP was species-specific for the primer sets we designed (Fig. 3). We were able to clearly and consistently identify pollen grains for each of the 3 species, while non-target pollen produced consistently negative results (see below). Mixing pollen grains in a single reaction also demonstrated a sensitive and robust capacity of LAMP to specifically detect the presence of small amounts of DNA (approximately three genomes) in environmentally relevant situations (Fig. 3.4).

On occasion, LAMP produced what appeared at first sight as potential false-positive results, since DNA amplification could be detected in negative control lanes of some gels stained with ethidium bromide (Fig. 3.5). However, close inspection of these lanes clearly shows different banding patterns which differ from those of positive controls (Fig. 3.6).

Discussion

We have demonstrated a simple but robust method for the quick, isothermal amplification of specific DNA sequences in pollen, with reliable sensitivity down to the level of a single pollen grain.

Our method avoided DNA extraction or purification, making it possible to reliably work with exceedingly low amounts of DNA template for the amplification reaction. The male gamete in a single pollen grain of the species utilized contains only three genome copies, which are easily lost even with the most careful extraction protocols. Keeping the whole pollen grain in the reaction tube insured that all DNA in the sample was

retained. The simple adaption of Chen et al.'s protocol, involving the permeabilization of the pollen cell wall with NaOH, subsequently neutralized for the reaction, operates well for all plant species tested so far, something also noted originally by Chen and collaborators in the case of PCR (Chen et al., 2008a). Thus the method has a clear potential for wide applicability across biological taxa.

LAMP produces approximately 10^9 copies from a few copies of a template DNA sequence in less than an hour and thus the potential for amplicon contamination is high. To limit this increased danger in biomedical applications and to assist with high-throughput instrumentation development various fluorescent reporters have been employed (Curtis et al., 2009b; Mori et al., 2006; Tomita et al., 2008). While this is undeniably essential for any high-throughput ecological study, separation by gel electrophoresis continues to be essential at least until discerning primer sets and reaction conditions have been carefully optimized. Since LAMP requires four to six primers for amplification, the potential for specificity is significantly raised compared with PCR, which requires only two primers. On the other hand, the complexity of the series of oligonucleotide interactions involved in the LAMP amplification can also lead to potentially confusing results if primer design is not performed carefully. Primer design and rigorous testing in the lab is therefore a precondition for any widespread deployment of LAMP in ecological situations. We have gained experience from many primer sets and several species, and have noted the potential for non-specific behavior, including primer-dimer amplification and cross-species mispriming. While some of these effects can be attributed to the choice of target for amplification (shared DNA sequences across specimens, whether by homoplasy or synapomorphy), other sources of possible confusion stem from the complexity of the LAMP reaction itself.

In Figure 5 we present examples of such possible sources of confusion in the use of LAMP in ecological situations. In particular, LAMP is often used as a source of presence/absence data, for example in pathogen detection under medical (Han, 2013) and phytopathological (Oya et al., 2008) situations. In these cases, the only observation, frequently through fluorescence methods, is the amplification of DNA in large amounts, without regard for the specifics of the DNA sequences amplified. "False positives" with LAMP have been recorded in the literature (Curtis et al., 2009a; Lee et al., 2009; Villari et al., 2013) and personal communications suggest that this is a problem occurring more commonly than reported.

In our experience, it is possible to distinguish between these two sources of amplification signal once amplicons are resolved through gel electrophoresis, and provided the primer set is adequately designed and tested for the desired application. Thus we show in Figure 6 how specific amplification of the target sequence results in amplicon sizes in a pattern that is identical between field pollen samples and purified DNA from the target species yet differ from non-specific amplification.

Annealing temperature gradients, primer concentration curves and time-series for amplifications are obvious starting points for optimizing LAMP reactions. In addition,

the integration of sequence-specific fluorescence probes resulting in a visual signal can also diminish the possibility of false-positives (Curtis et al., 2009b; Mori et al., 2006).

We continue to study and resolve the various barriers for the eventual use of LAMP in general ecological situations, but from the evidence accumulated so far, it is clear that primer design and cross-species testing is of paramount importance in the proper application of this highly promising.

DNA amplification using LAMP is a method with fast-growing popularity for field applications(Sirichaisinthop et al., 2011; Tao et al., 2011), since it avoids several of the steps necessary in the more traditional PCR method. Coupled with the simple, extraction-free protocol presented here, this method has the potential for a wide diversity of ecological applications. In addition, LAMP reagents are relatively inexpensive: in our protocol, the cost of a determination lies between 0.80 and 0.90 USD, and we expect this cost to drop dramatically as bulk prices and more competition in enzyme production reduces premium prices in the reagents we currently use.

Single-pollen grain LAMP has the potential to enable a much finer level of resolution in population genetic studies, provides a method for continuous and detailed monitoring of the aerobiology of diverse ecological situations (e.g. genotype-specific pollen loads of allergenic interest, cross-pollination studies, etc.), and promises many other applications. These potential applications will rely on the careful development of primer design and testing strategies, as well as the development of simple and inexpensive detection methods and instruments suitable to field situations.

Materials and Methods

Plant material and DNA Extraction

Pollen and tissue samples were collected in Berkeley, California, in 2012 and 2013. To show the viability of the method, three plant species were chosen to cover a wide range of spermatophyte phylogeny, as well as based on their availability and interest from palynological and allergenicity viewpoints. One important consideration was also the availability of sufficient genomic information in public databases to allow for effective primer design. The chosen species were sunflower (*Helianthus annuus*), ginkgo (*Ginkgo biloba*) and birch (*Betula pendula*). When needed, purified DNA was obtained using the DNeasy Plant Mini Kit from Qiagen (Gaithersburg, MD, USA). Purified DNA was obtained from massed-pollen samples, leaf- radicular apical meristems or bud tissues.

Pollen preparation

Chen et al.'s (2008) protocol for single-pollen grain PCR (Chen et al., 2008a) was adapted to prepare pollen grains for LAMP. This method allows for extraction-free

protocols, removing a time-consuming step in usual DNA amplification reactions. Access to genomic DNA inside the impervious pollen cell wall was achieved through a simple permeation protocol as follows: individual pollen was hand-picked under a dissecting microscope utilizing a new 25G (0.51 mm O.D.) hypodermic needle as a spatula and deposited into 0.5mL PCR tubes containing 1µl of 0.1M NaOH. Experimenting with various tools for this operation, it was found that the surgical steel and sharpness of hypodermic needles is ideal for the task. To avoid static electricity, we avoided plastic in the process, except for the final PCR tubes. Electrostatic charges in all tools, containers and tubes were removed by touching a line of grounded copper wire set around the microscope stage. Tubes were centrifuged briefly and the number of grains in each counted before incubating them for 17.5 min at 95°C in a standard PTC-100 thermocycler with a heated bonnet (MJ Research, Watertown, MA, USA). Tubes were cooled and briefly centrifuged to retrieve condensates before adding 2µl of a neutralizing buffer to each tube. The neutralizing buffer consisted of 0.2M Tris-Cl and 1mM EDTA at pH 8. The endpoint pH of these ratios of NaOH to neutralizing buffer was measured to be pH 8.2 at 21^{o}C.

Primer Design

Loop-mediated isothermal amplification (LAMP) was performed according to Nagamine et al. (Nagamine et al., 2002) using a set of six primers for each species. The first step in primer design was based on literature searches to identify sequences of DNA corresponding to unique proteins which were suspected to be diagnostic of each species. Genbank sequences were then sought for those sequences and tested *in-silico* through BLAST searches (Altschul et al., 1990), alignment analysis and further primer-set design. Chosen proteins were as follows: for sunflower, the 11S seed storage protein (Yonder Haar et al., 1988); for ginkgo, the antifungal peptide Ginkbilobin (Sawano et al., 2007) and for birch the major allergen Bet1v (Schenk et al., 2006). Forward and backward inner primers (FIP and BIP), forward and backward outer primers (F3 and B3) and forward and backward loop primers (F-Loop and B-Loop; REF), were designed using LAMP Designer Software (Premier Biosoft, Palo Alto, CA, USA) according to the conditions described by Notomi et al. and Nagamine et al. (Nagamine et al., 2002; Notomi et al., 2000b). Various primer sets suggested by the software were queried using NCBI BLAST to avoid cross-species homology (Altschul et al., 1990). As multiple isothermal annealing sites involved in LAMP make this simple BLAST approach insufficient to ensure specificity, further refinement in primer design was developed by iterative trial and error of primer-sets. Primer-set optimization was performed using purified genomic DNA. The primer sets resulting from this iterative process are shown in Table 1. Primers were synthesized by Eurofins Operon (Huntsville, AL, USA).

LAMP Reactions and Detection

For LAMP amplification, each reaction contained the following: 1.6 µM each of FIP and BIP, 0.2 µM each of F3 and B3, 0.8 µM each of F-Loop and B-Loop, 0.2 mM of each dNTP, 2.5µL of a 10X Isothermal Amplification Buffer (1X concentrations equal to: 20 mM Tris-HCl, 10 mM, $(NH_4)_2SO_4$, 50 mM KCl, 2 mM $MgSO_4$, 0.1% Tween® 20 at pH 8.8) and 16 units of Bst 2.0 Warmstart Isothermal Amplification Enzyme (the last two Reagents obtained from New England Biolabs, Ipswich, MA, USA).

To obtain the final reaction volume, 22µL of LAMP cocktail was added to each tube containing the permeated and neutralized pollen (i.e., 3 µL) and incubated at 63°C for one hour.

LAMP products were separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide and visualized on an ultraviolet transilluminator.

Acknowledgements: The authors would like to thank Cristian Orrego Benavente, Elena Alvarez-Buylla, Alma Pineyro and David Quist for their comments.

Figure Legends:

Table 3.1: Primers for the 3 species chosen. FIP and BIP primers are not continuous within the accession since part of the oligonucleotide corresponds to the complementary 3′ to 5′ strand and therefore two position coordinates are given. All coordinates are from the 5′ end.
Figure 3.1: LAMP Reaction for Bet1v. W: Water Control, L:100bp Ladder
Figure 3.2: ImageJ Analysis of positive lanes from Figure 1. Lanes 1-4: single birch pollen, Lane 9: isolated DNA positive control
Figure 3.3: Specificity for 3 species of pollen amplified with 3 different LAMP primer sets. L: 100bp Ladder, T: Positive control with isolated genomic DNA from each species, W: Water negative control
Figure 3.4: LAMP Reaction for Bet1v with multiple species present in each reaction. L: 100bp Ladder, W: Water Control
Figure 3.5: Examples of non-specific amplification. L: 100bp Ladder. Lanes 1,2,7,8,11 and 12 demonstrate positives while the remaining are non-specific amplifications.
Figure 3.6: ImageJ analysis of lanes 1-7 of Figure 5. 1. 100bp Ladder, 2-3. Positive amplification, 4-7 non-specific amplification

Table 3.1

Birch X15877, Birch mRNA for pollen allergen BetvI.	
FIP (38bp)	^{480}5′-AAGTGTCTCGCCCATTTCTT^{421}GGTGACCATGAGGTGAAGG-3′
BIP (37bp)	^{487}5′-GCCGTTGAGAGCTACCTC^{558}ATGTTCGAGACGACACAAGTTA-3′
F3 (22bp)	^{385}5′-TCCATCTTGAAGATCAGCAACA-3′
B3 (22bp)	^{589}5′-TGAACACTGCAACCCATTATTG-3′
Floop (21bp)	^{460}5′-TACTTGCCTTAACCTGCTCTG-3′
Bloop (21bp)	^{511}5′-CACTCCGATGCCTACAACTAA-3′
Sunflower M28832, Sunflower 11S storage protein (G3-D1) DNA, complete cds.	
FIP (39bp)	^{1797}5′-CGTCGCTTGCTCTTGTTG^{1720}AATGTCGGACAAGACCTTCAA-3′
BIP (37bp)	^{1805}5′-GCCAACAACAAGAGCAGC^{1882}ACTTCATGCTGCAGATGGT-3′
F3 (19bp)	^{1701}5′-CCAGAGAGGCCACATTGTT-3′
B3 (20bp)	^{1910}5′-TGGGAAGGGTTGTCAATGTT-3′
Floop (18bp)	^{1768}5′-AGCGTCTGTCTTGTGGTG-3′
Bloop (18bp)	^{1845}5′-GAGCAACGGTGTGGAAGA-3′
Ginkgo DQ496113, Ginkgo biloba ginkbilobin-2 precursor, mRNA, complete cds.	
FIP (36bp)	^{419}5′-CACCGATAGCGTTGTTGC^{358}CACTGCTTGCCTCTCCAA-3′
BIP (40bp)	^{433}5′-CGTCGACTGCTTCATCCA^{499}TCTGCTGACTAGAGATGGAAGA-3′
F3 (22bp)	^{325}5′-GACATGTAAGCAGTCCATCTCT-3′
B3 (22bp)	^{523}5′-AAGACCATCTGGGAGAGACTAT-3′
Floop (22bp)	^{398}5′-TAGAGAAGATGCGATTGACGAG-3′
Bloop (21bp)	^{455}5′-GAGCAGAGGAGCTTCTAATCC-3′

Figure 3.1

Caption: LAMP Reaction for Bet1v. W: Water Control, L:100bp Ladder

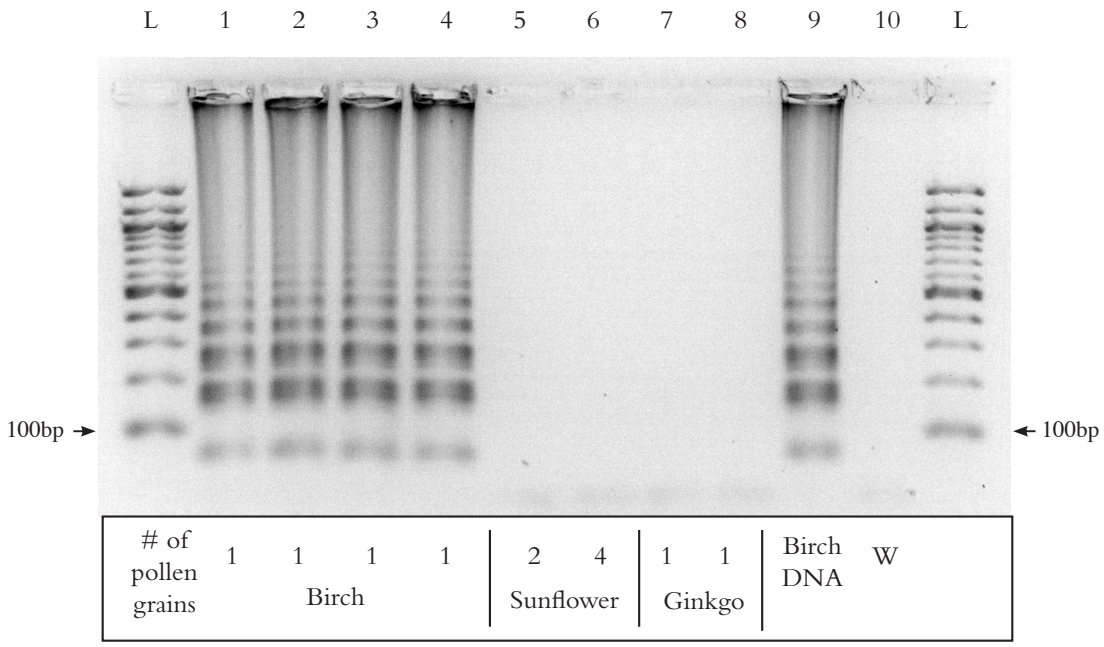


Figure 3.2
Caption: ImageJ Analysis of positive lanes from Figure 1. Lanes 1-4: single birch pollen, Lane 9: isolated DNA positive control

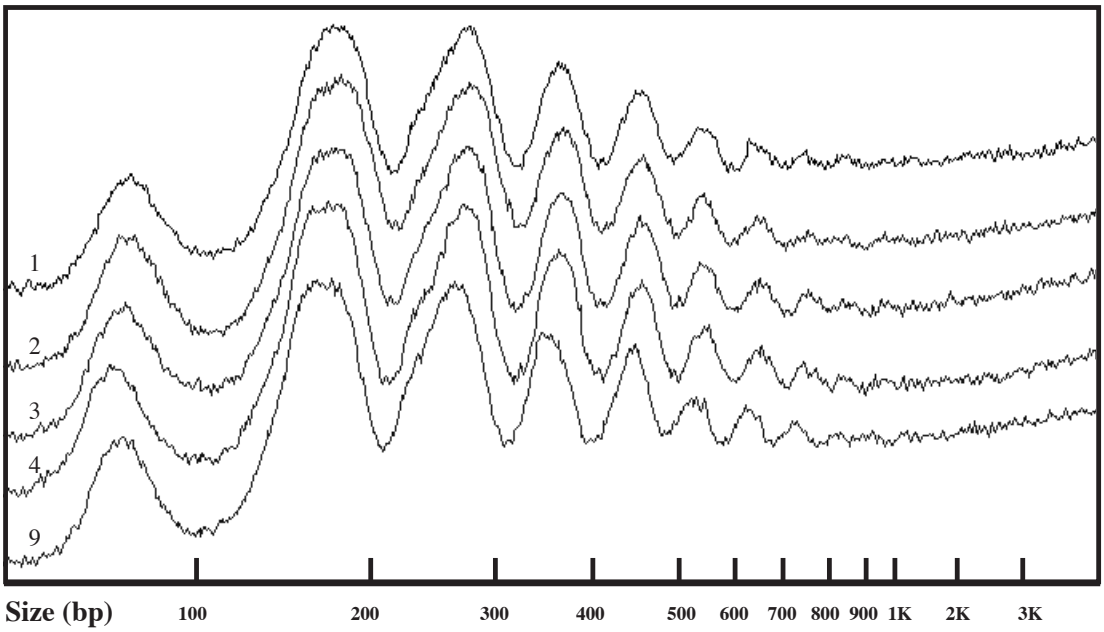


Figure 3.3

Caption: Specificity for 3 species of pollen amplified with 3 different LAMP primer sets. L: 100bp Ladder, T: Positive control with isolated genomic DNA from each species, W: Water negative control

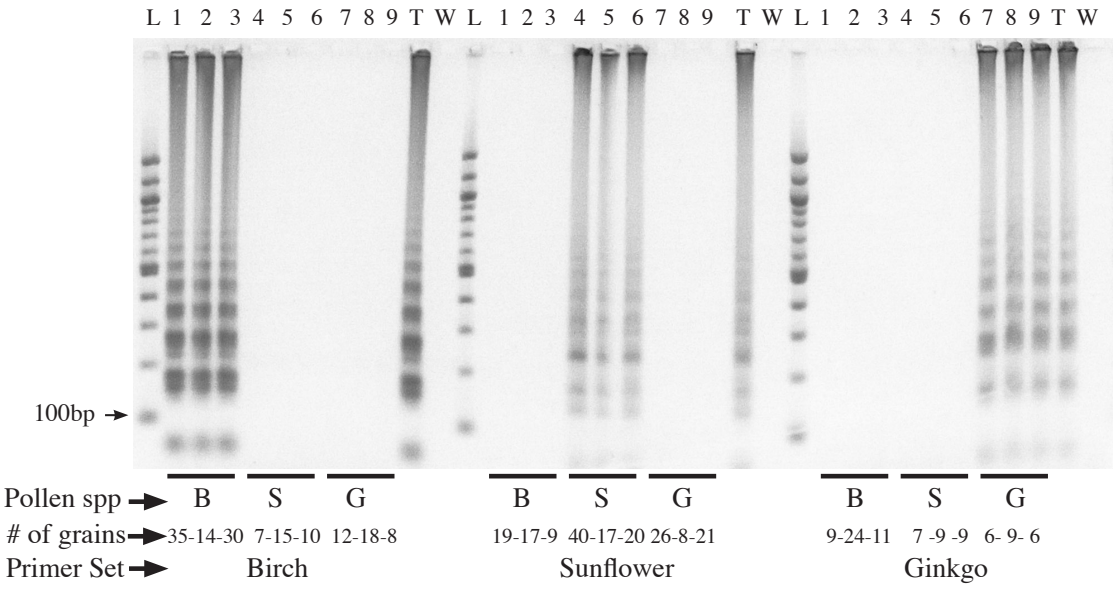
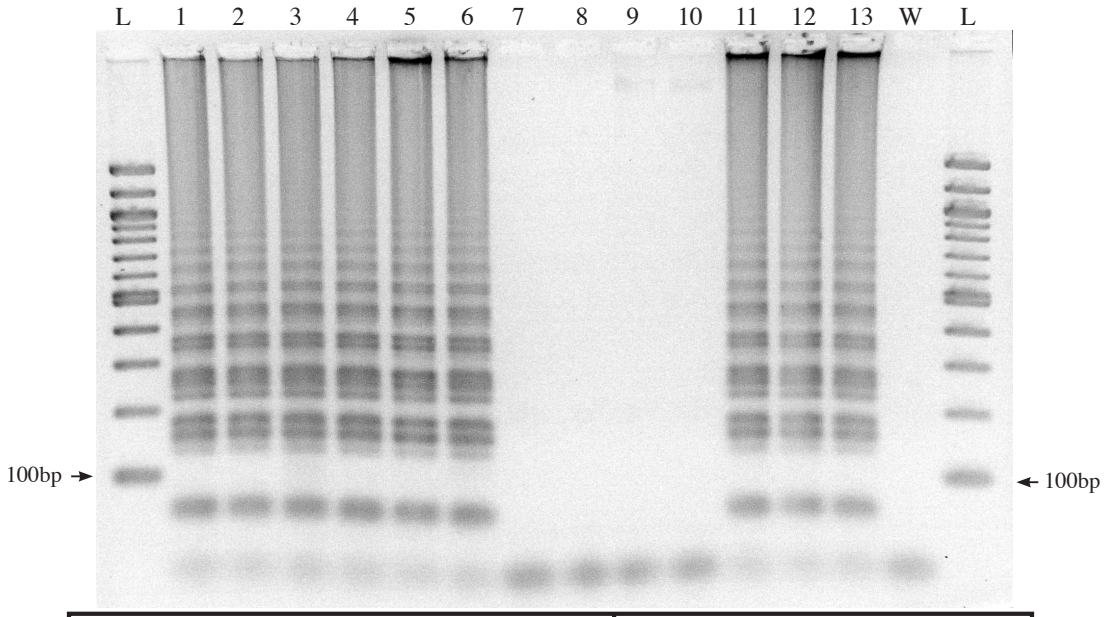


Figure 3.4

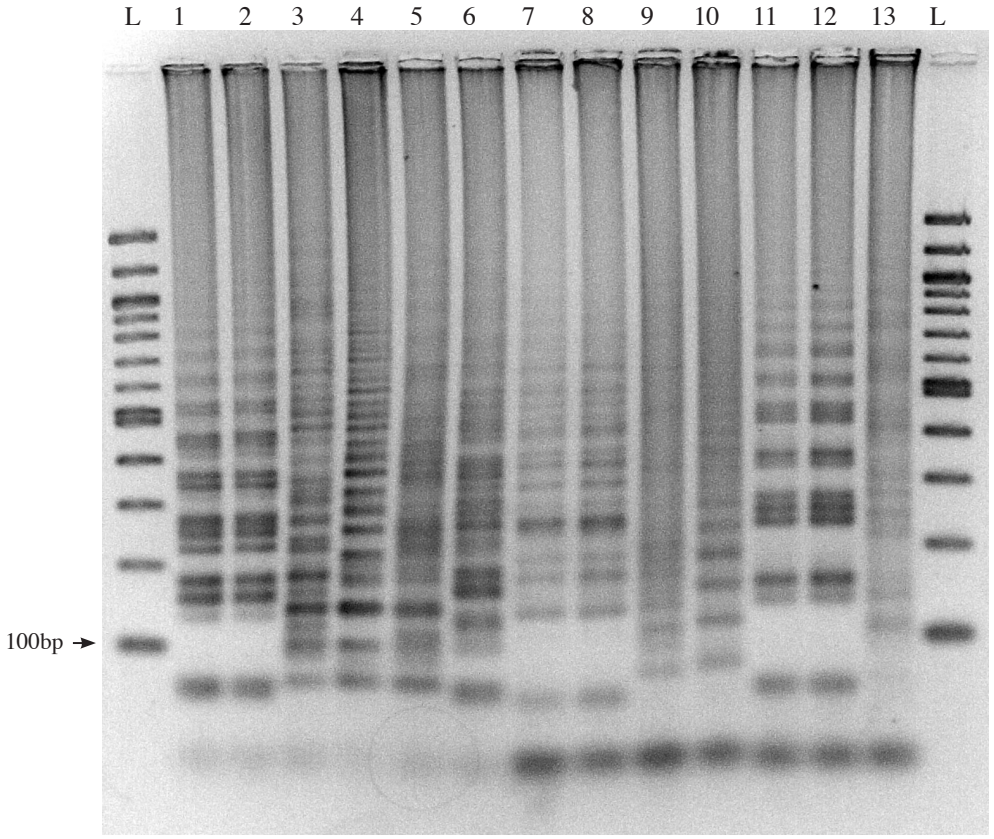
Caption: LAMP Reaction for Bet1v with multiple species present in each reaction. L: 100bp Ladder, W: Water Control



	Number of Pollen Grains								μL of Template DNA					
Birch	1	1	1	1	5	15	0	0	0	0	1	1	2	0
Sunflwr	4	5	5	3	5	18	5	9	1	1	1	1	0	0
Ginkgo	7	1	5	7	11	40	14	12	1	1	1	1	0	0

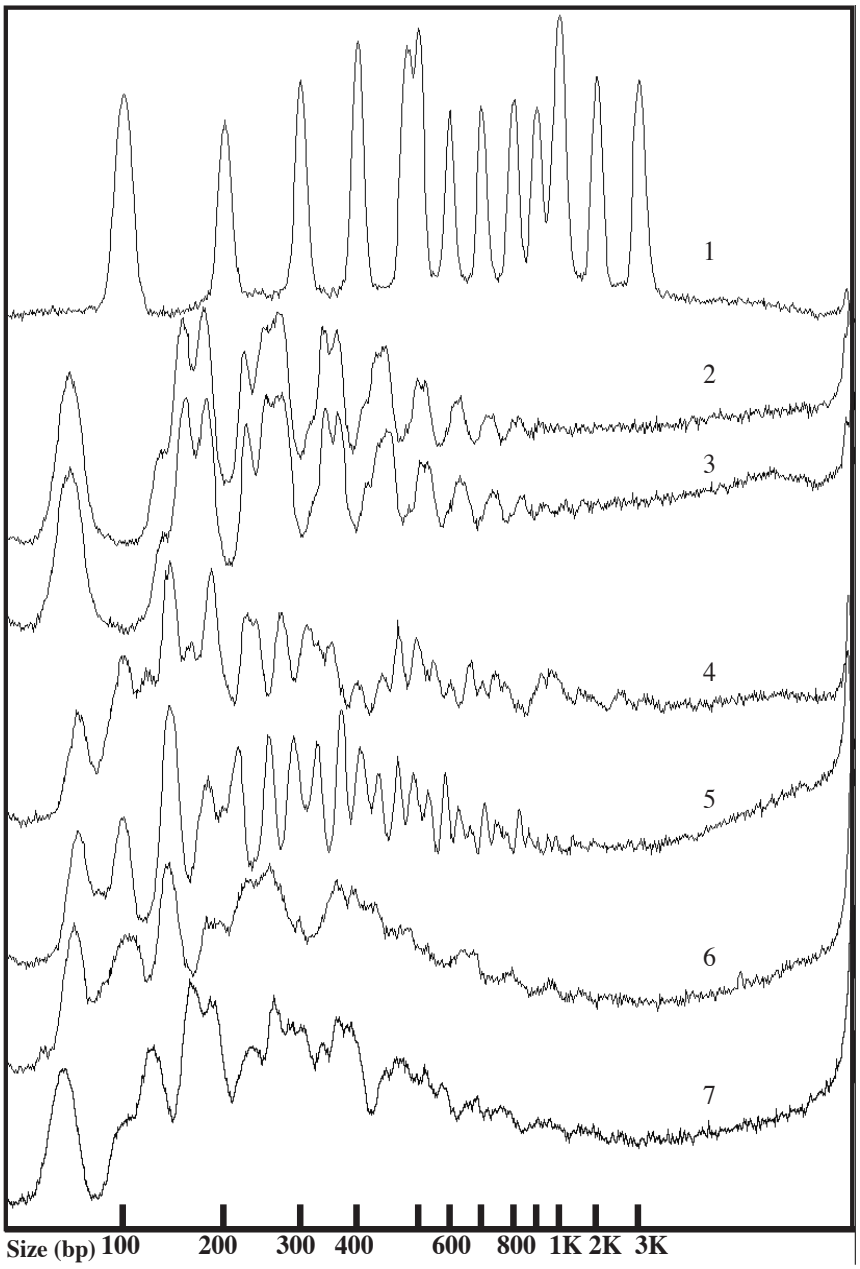
Figure 3.5

Caption: Examples of non-specific amplification. L: 100bp Ladder. Lanes 1,2,7,8,11 and 12 demonstrate positives while the remaining are non-specific amplifications.



# of pollen grains or μL template	5	2μL	1	14	2	0	19	2μL	8	3	7	8	17
species	B	B	S	G	G	-	S	S	G	G	G	G	B
	Bet1v (Birch) Primer Set						11s Seed Storage (Sunflower) Primer Set				Ginkbilobin (Ginkgo) Primer Set		

Figure 3.6
Caption: ImageJ analysis of lanes 1-7 of Figure 5. 1. 100bp Ladder, 2-3. Positive amplification, 4-7 non-specific amplification



Chapter 4: *submitted and under review by Aerobiologia*
A Fluorescent LAMP Method for the Field-Based Specific Detection of Pollen Grains

By Ali Bektaş and Ignacio Chapela

Abstract

Loop-Mediated Isothermal Amplification is being rapidly established as the preferred method for field-based, rapid and cost-effective detection of DNA sequences. We have previously demonstrated the applicability of this method for pollen from a variety of plant species with a resolution of down to a single grain. Although LAMP is increasing in popularity a hindrance of current methods for diagnostic purposes remains the prevalence of non-specific amplifications. In order to effectively deal with this problem a variety of fluorescent probes and oligonucleotides have previously been employed. Here we demonstrate one of these methods in the service of detecting pollen without the danger of false positives. We also establish its lower limits of detection and efficiency. In addition, we show that this method does not lose robustness for pollen of at least 8 years of age.

Introduction

The use of DNA sequences for detection of life-forms in a variety of environments, ranging from pathogens and disease agents to beneficial and endangered species, continues to develop as a key methodology for areas such as medicine, archaeology, ecology, forensics and many more. While much progress has occurred in terms of accuracy, sensitivity, coverage and even cost for methods of DNA sequence determination, there is an area that has only recently received attention: the use of DNA analysis methods directly in the field, especially under conditions of minimal- or no-laboratory infrastructure, as defined by Abou Tayoun (2014). Such are the conditions found in the large majority of ecological situations outside the urban centers of a few countries in the industrialized world. Furthermore, much of the progress in field-based molecular DNA analysis has taken place in life-forms of medical and veterinary interest, with much less attention paid to other applications. The equivalent of point-of-care diagnostics, much advanced in medicine, is missing for many other environmental applications, despite the potential impact of such approaches.

An ecological tool for use in direct-field situations requires similar standards to the methods and devices envisioned for medical uses in the developing world. The World Health Organization defined the characteristics of an ideal diagnostic tool for

direct field use in the developing world with the acronym "*ASSURED*", meaning that the methods should encompass the following characteristics (our adaptation from Urdea et al. (2006): *A*ffordable by those interested/impacted; *S*ensitive, with few false negative results; *S*pecific, with few false positive results; *U*ser friendly, requiring only minimal training to obtain results; *R*apid; *E*quipment-free; with methods; and with results *D*elivered as directly as possible to those who need it (Urdea, 2006). These same characteristics should be found also in a tool that could be used for ecological (i.e. non-medical) situations.

Using airborne pollen as a model, we seek to develop methods with *ASSURED* characteristics as described above, but with usefulness in a broad set of ecological situations, not only those of medical interest. After considering the wide palette of methodological options, we have identified the Loop Mediated Amplification reaction (LAMP) as the centerpiece for this goal (Bektaş and Chapela, 2014). In addition to the *ASSURED* properties, LAMP enables other requirements that are important for field application in an ecological context, particularly the removal of toxic materials and reagents. We have demonstrated the utility of LAMP for the specific detection of pollen grains down to a very high level of sensitivity, a single pollen grain (Bektaş & Chapela, 2014).

Here we continue our establishment of LAMP as a field-appropriate method to identify biological particles from a variety of environmental media. Using pollen as a model, we demonstrate the usefulness of fluorophore-quencher-oligomer DNA probe for the visualization of LAMP amplification, a method that obviates the use of gel electrophoresis as well as the use of toxic dyes for DNA visualization. This capacity further facilitates the use of this method in the field as a one-step/one-tube method, without the requirement for transfer of materials (which require specialized training), electrophoresis equipment or toxic waste management. In addition, here we explore the usefulness of the method for various numbers of pollen particles per sample, establishing patterns of reaction efficiency, as well determining the effect pollen grain age on the efficacy of the method.

Materials and Methods

Pollen and DNA Preperations

Maize pollen from 2014 and 2010 was collected in Brentwood, CA while pollen from 2006 was grown in the greenhouse. All plant genomic DNA was extracted in 2013 from leaf material collected that same year using a DNEasy Plant Mini Kit from Qiagen (Gaithersburg, MD, USA). Pollen grains picked underneath a dissecting microscope with the aide of a hypodermic needle were prepared prior to amplification following a modified protocol by Chen *et al.* (2008); heating for 17.5 min at 95°C in 2µl of 0.1M NaOH followed by centrifuging and cooling the tubes and adding 4µl of a neutralizing

buffer (0.2M Tris-Cl and 1mM EDTA at pH 8) to bring the pH to 8.2 at 21°C.

Primer Design

All oligonucleotides were designed from a Genbank sequence for the Maize alpha-zein protein and using a trial version of LAMP Designer Software (Premier Biosoft, Palo Alto, CA, USA), modified according to the fluorescent detection method outlined by Curtis *et al.* (2009) and synthesized by Eurofins Operon (Huntsville, AL, USA).

Maize mRNA for 10kDa zein (Accession Number: X07535)	
FIP (Forward Internal Primer)	^{330}5′-CATCATGCTCGGCAAGACCA^{264}GCCTAACATGATGTCACCAT-3′
BIP (Backward Internal Primer)	^{344}5′-TGCCACAATGTCACTGCGAG^{412}GTTGAACATGAATGGTAACTG-3′
F3	^{244}5′-ATGATGCCACAGATGATGAC-3′
B3	^{478}5′-CTATCTAGAATGCAGCACCAA-3′
F-Loop (Forward Loop Primer)	^{309}5′-TGGTGACATCATGCTCGG-3′
B-Loop Fluoro (Fluorescent Backward Loop Primer)	^{365}5′-[6-FAM]CCGTCTCGCAGATTATGCT-3′
B-Loop Quencher (complementary to B-Floop Fluoro)	5′-AGCATAATCTGCGAGACGG[BHQ1a-Q]-3′

LAMP Reaction

Each 25µl LAMP reaction was prepared with 2.5µl 10x LAMP Reaction Buffer (1X concentrations: 20 mM Tris-HCl, 10 mM, $(NH_4)_2SO_4$, 50 mM KCl, 2 mM $MgSO_4$, 0.1% Tween® 20 at pH 8.8) and 2µl (16units) BST 2.0 Warm Start DNA Polymerase (New England Biolabs, , Ipswich, MA, USA), 0.2mM of each dNTPs , 2µl 5M Betaine (Sigma,), 1.6µM each of FIP and BIP, 0.2µM of F3 and B3 and 0.8µM each of F-Loop and B-Loop Fluoro. Volume was brought up to 19µl with the addition of 10.7µl of water for each reaction to result in 25µl after the addition of 6µl sample (pollen in 2µl 0.1M NaOH and 4µl Neutralizing buffer). The reaction was carried out for 1hr at 63°C within a PTC-100 Thermocycler (MJ Research,Watertown, MA, USA). Following the reaction 1.5µl of 50µM of the quencher oligonucleotide was added and mixed by vortexing.

Agarose Gel Electrophoresis and Fluorescence Detection

Results of the reaction were visualized both by observing the fluorescence of the tubes over a UV transilluminator as well as running products on a 2% high resolution agarose gel imaged again with a UV transilluminator both before and after ethidium bromide staining.

Results

Specific Detection of Amplification Products: Towards a One-Step/One-Tube System

In order to provide a one-step/one-tube method of utility for the field ecologist, we sought to remove the necessity of using electrophoresis for the visualization of the accumulation of DNA as a result of specific amplification. This is a crucial goal, since it is well known that some degree of non-specific amplification occurs in LAMP for reasons still unknown, which can result in false positives (Bektaş and Chapela, 2014; Iwamoto et al., 2003; Villari et al., 2013)

To achieve this goal, we adapted the method first described by Curtis *et al*. (2009) to be used in our pollen-grain amplification. This method utilizes a flurophore-quencher-adapted oligomer of DNA as a probe, which hybridizes with sequence-specificity to the appropriate amplification results after the LAMP reaction has taken place. Our adaptation of the method proved fully appropriate to the goal of performing the detection of specific LAMP amplification in a one-step/one-tube process. Importantly, when compared with the resolution of an electrophoresis, the probe was able to discern between false-positive reactions and real positives (Figure 4.1). Without exception, this probe approach consistently produced a fluorescent signal in the test tube every time we observed real positives, as evidenced by their electrophoretic banding patterns (Fig. 1; Bektaş & Chapela, 2014). On the other hand, very consistent negatives (i.e. no fluorescence) were observed using the probe method, even for tubes where non-specific amplification occurred either as isolated bands (Lane 6 in Figure 4.1) or as non-specific complex banding patterns (Lane 9 in Figure 4.1). While Figure 1 shows one example of these results, we have observed extremely consistent results over several hundred reactions.

Template Concentration and Method Sensitivity

Pollen grains provide a unique opportunity to test the level of sensitivity of a method with extreme precision, since it is possible to introduce a known number of pollen grains into a reaction and record the outcome. Using this capacity, we explored the efficiency of the LAMP reaction at very low concentrations of DNA; while we can consistently obtain positive LAMP reactions from single pollen grains, this does not happen every time, raising the question of robustness and reliability of the method, as opposed to its sensitivity.

Three separate runs were conducted of fourteen repeats of LAMP on single, 10, 30 and 100 grains within each reaction and the results observed through fluorescence. By using multiple repeats of the LAMP reaction performed with various known numbers of pollen grains per reaction, we were able to construct a “dose-response” curve for the LAMP (Fig. 2). This curve displays the expected logarithmic behavior with relatively low

success *rates* for one or a few pollen grains, but rapidly escalating in success of detection to eventually tend asymptotically to a plateau of 100% success rate. The 50% success rate point defining the dose-response curve lies at 5 pollen grains per tube (Fig. 2). The general outline of this curve as well as the specific parameters defining it were consistently obtained from the results of three separate runs, and 168 individual reactions, of this experiment, suggesting that these results are robust for the given reaction conditions. The equation of a logarithmic best-fit to the data was $y = 0.1813 \ln(x) - 0.0248$, with $R^2 = 0.91655$.

A useful comparison of this pollen-grain dose-response curve was obtained by producing a similar dose-response curve for purified maize DNA extracted from pollen. Here again, results were consistent over four repeats of the experiment, giving a best-fit logarithmic equation from these data of $y = 0.1855\ln(x) + 1.1243$, with an $R^2 = 0.94234$. In other words, very similar parameters were obtained from this comparative curve of purified DNA and from pollen grains without extraction. To our knowledge, this is the first evaluation of LAMP reaction efficiency in field-relevant biological samples.

Effect of Pollen Grain Age on Reaction Efficiency

Maize pollen stored for various periods of time in the dry dark at room temperature produced LAMP amplification results that were indistinguishable from each other. Over an 8 year period, no decline or increase in the efficiency of LAMP was observed for these grains, using the LAMP reaction conditions described here.

Discussion

LAMP has emerged over the last ten years as a potentially powerful method for use in field conditions for a variety of applications, particularly in human and veterinary medicine. Less effort has been devoted to the use of this powerful method for other field applications, in a broader ecological context. A model for this kind of application is provided by aeropalynology. Obtaining a field-able method for the detection of specific DNA sequences in pollen has well-recognized practical usefulness for example for allergen monitoring, paleontological studies and floristic evaluation. In addition to this value pollen grains provide a valuable model of single biological particles that can be seen and manipulated for the fine development of methods that can be extended to much wider ecological applications. In short, this capacity would enable the possibility of performing analyses of any biological group in the environment, as long as there were microscopic DNA-carrying particles from that group in the air, or other fluids, such as water. This capacity would extend to any air-borne particle, not only pollen: spores, bacterial cells, skin-cells, insect fragments or similarly small fragments of any other life-form.

The criteria provided by the WHO in the context of medical diagnostics for use in the developing world (ASSURED, see Introduction above), represent a useful starting point for a field-abled method of wider ecological application. The characteristics of ASSURED, however, must be further expanded and detailed to a point where cost and ease of use, in addition to robustness, would allow a method for widespread adaptation. Our work aims towards providing such an expanded capacity in the detection of pre-determined, specific DNA sequences carried by air-borne particles.

Based on the work of Notomi *et al*. (2000), we have demonstrated that the LAMP-based method can be efficiently tuned to the point of application on one single particle per reaction, which would aid in the goal of miniaturization. This in turn opened the possibility of inexpensively analyzing large numbers of ecological samples with particle-level resolution and specificity. In addition, our single-pollen grain analysis also furthers the goal of a method that could be performed in one single tube ("one-tube"), and potentially through a single step of manipulation ("one-step"). This goal would obviate the need to transfer either sample- or reagent solutions, which is one of the steps in any processing which prevents easy field use since it requires delicate operator expertise and specialized instruments and reagents such as micro-pipettes and distilled water.

By using a hybridization-fluorescence method based on LAMP, we have also shown the possibility of avoiding the relatively complex and costly instruments needed for electrophoresis resolution of DNA mixtures, or their visualization. This goal has been attempted through other methods in the past; for example, Mori et al. showed that the precipitation of pyrophosphate as a byproduct of the LAMP reaction could be visualized after precipitation through centrifugation. In our experience, such a method is unreliable because the amount of pyrophosphate needed in order to allow naked-eye visualization is simply not guaranteed by the reaction under all conditions leading to real amplification (low robustness), in addition to the equipment implied by the required centrifugation step.

A second method first introduced by Tomita *et al.* (2008) and tested in our lab relies on the chemical exchange of Mg^{++} divalent cations for Ca^{++} in the fluorophore calcein. The calcein molecule fluoresces several fold more dimly when bound to Mg^{++} and as it becomes bound to increasing amounts of pyrophosphate released from the synthesis of DNA through the LAMP reactions, the Mg^{++} is sequestered as covalently-bound Mg-pyrophosphate, thus releasing the quenching effect and increasing the calcein fluorescence to provide a signal for a positive reaction. This ingenious method has shown some promise, but in our extensive experience it proved to be too sensitive to minute differences in Mg^{++} concentrations, which are again variable to the degree that LAMP amplification can vary in its yield of amplified DNA ("lamplicon").

Finally, the risk of false positives caused by the above methods can be obviated using electrophoresis (Lee et al., 2009)and to a less reliable extent real-time detection of

DNA (Villari et al., 2013), but such additional steps are undesirable for a method with real potential for low-cost, field-compatible use.

The hybridization-fluorescence method presented here addresses all the problems mentioned above, and removes a series of hurdles to eventually achieve a practicable, ecologically-abled, ASSURED-compatible approach to DNA analysis at a real geographical scale. In our experience, this method is extremely reliable and reproducible, it produces a clear signal that can be easily detected with relatively simple optical capacity (light filtering), and it avoids the many pitfalls inherent in the practice with all other methods of which we are aware.

Although in our experience over several hundreds of assays the rate of false positives using our method has been literally nil, a question remained to be addressed: an estimation of false negatives. We approached this question by determining the rate of failure to detect a DNA sequence known to exist in a sample as a function of the number of copies of that DNA sequence. We find that despite our previous demonstration of effectiveness in the LAMP reaction to detect extremely low copy numbers of a DNA sequence, there is indeed a dose-dependence of the LAMP reaction.

Since we were able to visually count the particles used in each tube for a large number of LAMP reactions, we could parameterize such a dose-dependence function (Fig. 4.2). This is, to our knowledge, the first time such a detailed positive evaluation of the LAMP reaction has been achieved. Furthermore, because individual doses of pollen genomic DNA were positively delivered into each tube (confirmed by microscopic observation of each tube before the reaction), we were also able to determine that such dose-dependence function is indeed determined by biochemical parameters of the LAMP reaction, and not by probabilistic behavior displayed through the more usually performed dilution-response curves.

What our dose-dependence curves imply is that at very low count numbers, and particularly at the level of single-particle analysis, LAMP can be considered as a reliable (no false positives), if also not strongly sensitive (relatively high rate of false negatives) method for use in the field. A field-based method based on this technique would need to take this into account, with the obvious corollary that in order to be able to base any ecological statement on single-particle analysis using our LAMP method, a large number of assays must be performed, since relatively high levels of false negative determinations should be expected.

Significantly, the pollen-count curves of LAMP efficiency are practically identical to curves generated by dilution of extracted and purified DNA (Fig 4.2). This fact further supports the two hypotheses that (a) our non-extractive method for accessing pollen DNA (Chen et al., 2008b) is practically indistinguishable from an extraction procedure, and (b) there are dose-dependent processes in the LAMP reaction that can trigger a yes/no amplification cascade, as opposed to strictly dose-response effects. The latter hypothesis is important, because the alternative possibility, namely that the LAMP reaction could operate as a strictly dose-response reaction, would make this method

difficult to apply or interpret, particularly for field-based, low-copy-number DNA analysis. The LAMP method described here is therefore useful as a yes/no approach to the determination of presence/absence of specific DNA sequences in a single particle or many particles from a sample life-form, with all the positive qualities of an ASSURED- and ecologically suitable method.

We would like to thank Emmanuel Gonzaléz-Ortega and Michelle Katuna for their assistance in the laboratory and Elena Alvarez-Buylla and Cristián Orrego Benavente for their guidance.

This work was supported by UC MEXUS and the Peder Sather Grant Program.

Figure 4.1 LAMP reactions, tubes under UV excitation (panel A) correspond to lanes below them (panel B), 1-3 Unique Corn DNA Extractions, 4. Hazel DNA extraction, 5. Sunflower DNA extraction, 6. Ginkgo DNA Extraction, 7. Cedar DNA extraction, 8. Alder DNA Extraction, 9. Birch DNA extraction, 10. Water Control. L- Ladder

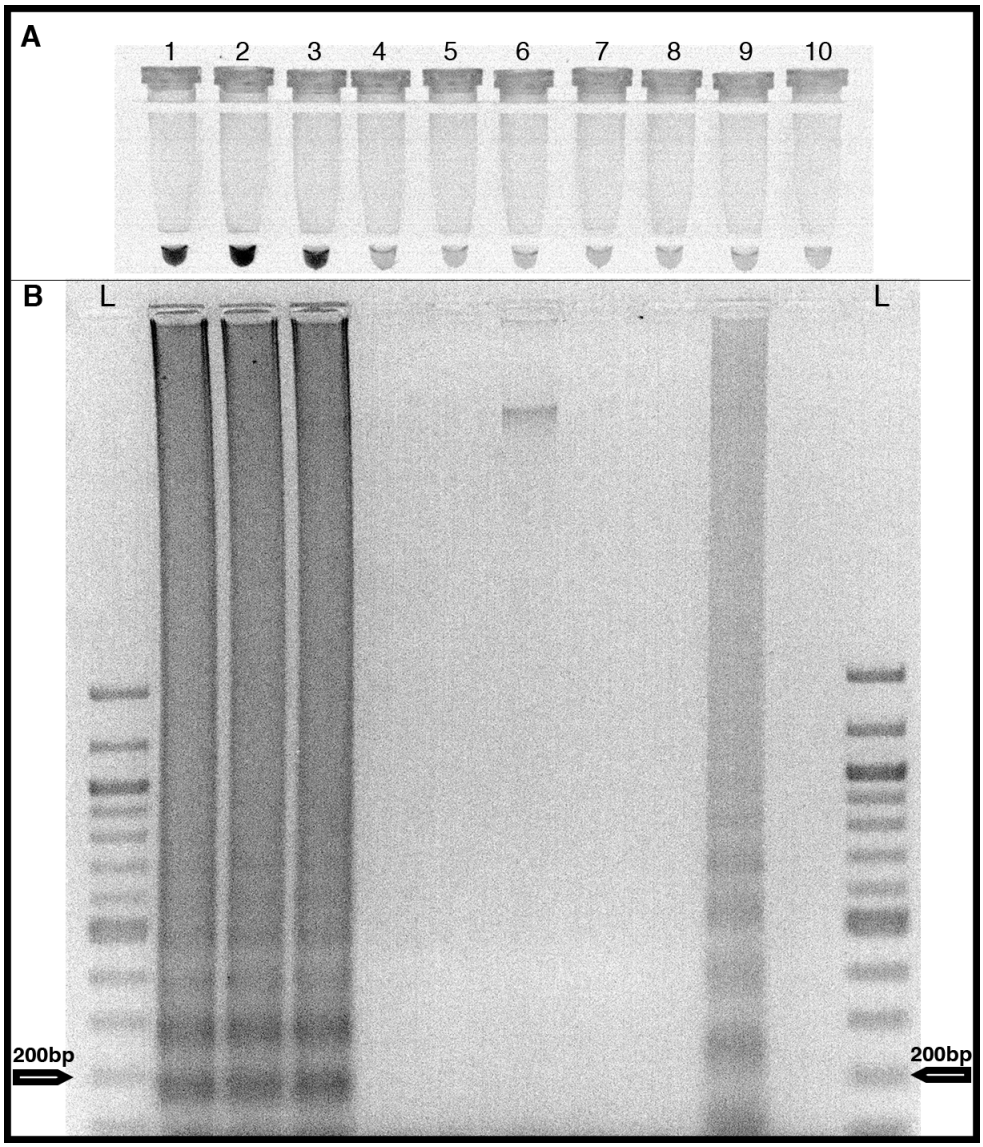
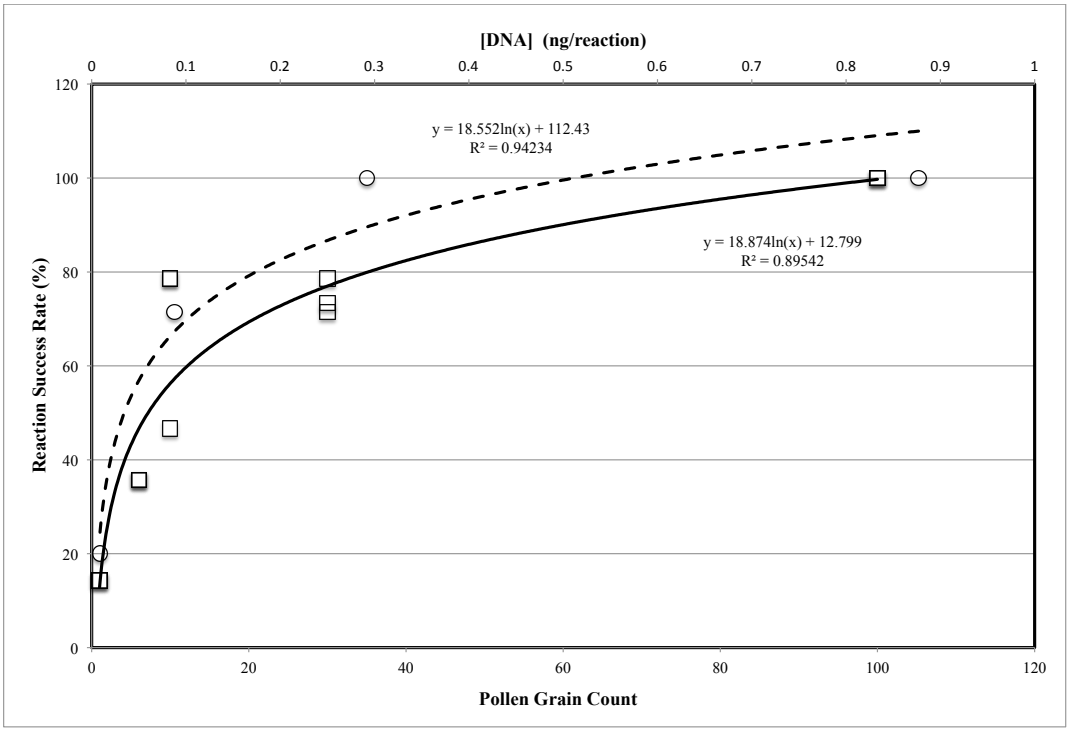


Figure 4.2 Trendline of pollen LAMP success rate against number of grains and ng of DNA



Box 4.1 Characteristics of the ideal diagnostic test for the developing world applied to LAMP, from Urdea et al.

ASSURED

- **A**ffordable by those at risk of infection.
- **S**ensitive (few false-negative results).
- **S**pecific (few false-positive results).
- **U**ser-friendly (simple to perform by persons with little training).
- **R**apid treatment at the first visit and robust use without the need for special storage.
- **E**quipment-free (that is, no large electricity-dependent instruments needed to perform the test; note that portable handheld battery-operated devices are acceptable, which differs from the criterion of the original authors).
- **D**elivered to those who need it

Chapter 5:
A Multiplex, Fluorescent Method of LAMP DNA Amplification for Detecting GMO Corn

By Ali Bektaş and Ignacio Chapela

ABSTRACT

Detecting, monitoring and mapping transgenic sequences in the environment has been a long-standing challenge in ecology. In addition to intense research interest, the capacity to specifically detect DNA sequences in the environment also has major economic and other socially important consequences. Given the level of social, economic and research interest in them, transgenic (GMO) DNA sequences stand as paradigmatic in this quest; such sequences further have the unique property of functioning as unequivocal markers in the environment, clearly showing the paths of gene flow between cultivated and wild life-forms, crops and their wild relatives, and across industrial and traditional forms of agriculture.

Although there have been a few proposals for field-based detection of transgenes, so far such capacity remains restricted to professional laboratories, private and public, academic and commercial. The analyses conducted in this manner, even in the field, can only perform a low number of determinations.

True mapping of transgenic DNA at the landscape level requires a method of DNA amplification that is robust and isothermal in order to be cost effective and field-based. We present here a method with these characteristics. Our multiplex fluorescent method is demonstrated for the detection of the p35s CaMV promoter frequently used in transgenic plants as well as the alpha-zein protein specific to maize, but is applicable to any other DNA sequence. The method uses Loop- Mediated Isothermal Amplification (LAMP) with visualization achieved using a fluorophore-quencher system of DNA hybridizing probes. We demonstrate the applicability of this tri-color method to transgenic corn, non-transgenic corn, and non-transgenic/non-corn species.

Introduction

Since the introduction of transgenic crops into global landscapes in 1987 (Seidler and Hern, 1988) there has been a growing concern about their unintended movement into non-transgenic crop varieties and food supplies. As the distribution of such so-called GMO crops expands, there has been a steady interest in developing detection, monitoring and mapping approaches to follow their expansion.

The common DNA detection technique has traditionally been based on the decades-old Polymerase Chain Reaction (PCR; Mullis et al., 1986). Initially used as a

reaction to detect single, short sequences of DNA that are diagnostic of GMOs, PCR evolved over the years to encompass longer transgenic sequences, sequences adjacent to the transgenic insert (Querci et al., 2009) and eventually multiplex reactions which can detect simultaneously more than one sequence in a single reaction. In a multiplexed reaction, a multiplicity of primer pairs are utilized to detect more than one DNA sequence at the same time. Although requiring greater care during primer design due to the delicate convergence of the annealing temperature of at least 4 oligonucleotides, multiplexing cuts detection time in at least half, making it ideal for large-scale detection and monitoring situations. Multiplex also reduces costs because it utilizes reagents more effectively. This has led to the development of primer sets and protocols to detect both internal reference genes for a variety of crops species and their transgenic events. There have been numerous publications outlining the PCR-multiplex detection of transgenic corn (Huang and Pan, 2004), soy (Hurst et al., 1999), canola (James et al., 2003), cotton (Kim et al., 2008), tobacco (Singh et al., 2007) amongst others.

The past decade has seen the development of a variety of novel isothermal DNA amplification methods. One central goal of isothermal amplification is to remove the technical barrier required by rapid temperature cycling, essential for PCR. Such techniques include rolling-circle amplification (Demidov, 2002), strand displacement amplification (Walker et al., 1992) and nucleic-acid sequence-based amplification (NASBA) (Deiman et al., 2002). One isothermal DNA amplification method of particular interest is the Loop-Mediated Amplification (LAMP) (Notomi et al., 2000c) due to its simplicity, robustness, resistance to inhibition factors and ease of management in laboratories with minimal equipment (Gill and Ghaemi, 2008; Bektaş & Chapela, 2015).

In contrast to PCR, LAMP utilizes 4 to 6 oligonucleotides and harnesses the 5’-3’ strand displacement activity of the polymerase utilized, DNA Polymerase I-Large Fragment, from *Bacillus stearothermophilus* (Bst). Based on its isothermal qualities, many applications of LAMP have been proposed for point-of-care delivery and field-based amplification. LAMP has also been utilized in the detection of transgenes with a few publications illustrating the amplification of the p35s CaMV promoter frequently used in transgenic modifications.

Being a rapid and isothermal technique there has also been the gradual emergence of protocols outlining the multiplexing of LAMP primer sets for more than one single DNA sequence. Although molecularly complex, employing upwards of 8 oligonucleotides, protocols utilizing multiplex LAMP have been published for parasites from mosquitos (Aonuma et al., 2010), foot-and-mouth disease virus (Yamazaki et al., 2013), Salmonella ssp. and Shigella ssp (Shao et al., 2011) and influenza (Mahony et al., 2013) amongst other microorganisms. No such methods, however, have yet been applied to GMO detection.

We present here the first use of a multiplex LAMP reaction to be applied, in the field, for the detection of transgenic DNA as well as its host species. The reaction described below presents the rapid, isothermal amplification of both the DNA

associated with the alpha zein protein endogenous to maize as well as that associated with the CaMV p35s promoter, in a single reaction. Furthermore, by utilizing a fluorescence-based reporting mechanism (Curtis et al., 2009a) we demonstrate a visualization method which requires minimal instrumentation, at the same time as it removes from the process DNA-visualization dyes which can be toxic and thus compromise their use in the field. The resulting method can therefore be considered as a field-ready approach to the detection, monitoring and/or mapping of GMO DNA in the field.

Results

We have confirmed the applicability of the two primer sets, one designed by us and the other from the literature (Fukuta et al., 2004) to act in concert with the fluorophore-quencher modification, first published by Curtis et al. (2009). By using Non-transgenic Maize DNA, Transgenic Maize DNA, transgenic soy DNA, cedar DNA and a Water control we were able to confirm the specificity of the primer sets.

Figure 1 illustrates, through its 4 panels, the primer sets at work (or lack of amplification) on the 4 different templates. The green fluorophore, fluorescein, is attached to the B-Loop primer for the alpha-zein protein and therefore only observable in reactions containing template form non-transgenic and transgenic maize DNA (Fig. 5.1, tubes and lanes 1-4). The red fluorophore, TexasRed, is attached to the B-Loop primer for the Cauliflower Mosaic Virus and therefore only observable in the reactions containing template from transgenic maize and transgenic soy.

Panel D shows the results of running the LAMP reactions through 2% agarose gel electrophoresis. In our previous work we have illustrated that LAMP reactions have specific banding patterns which correspond to the primer set utilized (Bektaş and Chapela, 2014) and one would expect Panel D to illustrate this with lanes 3 and 4 being a composite banding pattern of what is observed in Lanes 1-2 and 5-6. Upon first observation the banding patterns between all 6 positive amplifications appear similar, but the clear specificity of the fluorescently tagged B-Loop primer indicates beyond doubt that the LAMP products are in fact specific for the template they were designed for. In general, in the literature there appears to be a lack of investigations probing the exact nature of “LAMPlicons.” This is a clear necessity for all multiplexed LAMP reactions, including the one presented here.

Materials and Methods

DNA Preparations

DNA from 5% EU standard Transgenic Soy reference material (FLUKA,Sigma), transgenic and non-transgenic maize grown in the greenhouse, and cedar pollen collected on UC Berkeley campus was extracted in 2013 using a DNEasy Plant Mini Kit from Qiagen (Gaithersburg, MD, USA).

Primer Design

Two sets of oligonucleotides were used to develop this assay. The set detecting the maize-specific sequence for the 10kDA zein protein was developed using a trial version of LAMP Designer Software (Premier Biosoft, Palo Alto, CA, USA) (Bektaş & Chapela, 2015). The second set of oligonucleotides specific for the CaMV p35s promoter, frequently used in transgenic plants was designed by Fukuta et al. (2004). Both sets of oligonucleotides were modified according to the fluorescent detection method outlined by Curtis *et al.* and synthesized by Eurofins Operon (Huntsville, AL, USA).

Maize mRNA for 10kDa zein (Accession Number: X07535)	
FIP (Forward Internal Primer)	^{330}5′-CATCATGCTCGGCAAGACCA^{264}GCCTAACATGATGTCACCAT-3′
BIP (Backward Internal Primer)	^{344}5′-TGCCACAATGTCACTGCGAG^{412}GTTGAACATGAATGGTAACTG-3′
F3	^{244}5′-ATGATGCCACAGATGATGAC-3′
B3	^{478}5′-CTATCTAGAATGCAGCACCAA-3′
F-Loop (Forward Loop Primer)	^{309}5′-TGGTGACATCATGCTCGG-3′
B-Loop Fluoro (Fluorescent Backward Loop Primer)	^{365}5′-[6-FAM]CCGTCTCGCAGATTATGCT-3′
B-Loop Quencher (complementary to B-Floop Fluoro)	5′-AGCATAATCTGCGAGACGG[BHQ1a-Q]-3′
Cauliflower Mosaic Virus [GenBank accession No. V00141]	
FIP (Forward Internal Primer)	^{7243}5′-AGGCATCTTCAACGATGGCCTT^{7179}AAAGGAAGGTGGCTCCTACA-3′
BIP (Backward Internal Primer)	^{7245}5′-TGCCGACAGTGGTCCCAAAG^{7324}TTGAAGACGTGGTTGGAACG-3′
F3	^{7141}5′-TGCCCAGCTATCTGTCACTT-3′
B3	^{7364}5′-TCCCTTACGTCAGTGGAGAT-3′
F-Loop (Forward Loop Primer)	^{7221}5′-TCCTTTATCGCAATGATG-3′
B-Loop Fluoro (Fluorescent Backward Loop Primer)	^{7285}5′- [AminoC6+TxRed]AGCATCGTGGAAAAAGAAG-3′
B-Loop Quencher (complementary to B-Floop Fluoro)	5′-CTTCTTTTTCCACGATGCT[BHQ1~aQ] -3′

LAMP Reaction

Each 25µl LAMP reaction was prepared with 2.5µl 10x LAMP Reaction Buffer (1X concentrations: 20 mM Tris-HCl, 10 mM, $(NH_4)_2SO_4$, 50 mM KCl, 2 mM $MgSO_4$, 0.1% Tween® 20 at pH 8.8) and 2µl (16units) BST 2.0 Warm Start DNA Polymerase (New England Biolabs, , Ipswich, MA, USA), 0.2mM of each dNTPs , 2µl 5M Betaine (Sigma,), 1.6µM each of FIP and BIP, 0.2µM of F3 and B3 and 0.8µM each of F-Loop and B-Loop Fluoro. Volume was brought up to 22µl with the addition of 12.4µl of water for each reaction to result in 25µl after the addition of 3µl of extracted DNA. For reactions conducted directly on pollen grains 9.4 µl of water was added to pring the volume to 19 µl in anticipation of a 6 µl sample (pollen in 2µl 0.1M NaOH and 4µl Neutralizing buffer).

The reaction was carried out for 1hr at 63°C within a PTC-100 Thermocycler (MJ Research,Watertown, MA, USA). Following the reaction 1.5µl of 50µM of the quencher oligonucleotide was added and mixed by vortexing.

Agarose Gel Electrophoresis and Fluorescence Detection

Results of the reaction were visualized both by observing the fluorescence of the tubes over a UV transilluminator as well as by running products on a 2% high resolution agarose gel imaged again with a UV transilluminator after ethidium bromide staining.

Two separate filters from Chroma (Bellows Falls, VT) were utilized to observe emission spectra of the two separate fluorophores. For the fluorescein tagged B-Loop primer, part of the maize-specific primer set, a filter separating the 513-556nm wave length and for the TexasRed tagged B-Loop primer, part of the CaMV-p35s specific primer set, a filter separating the 600-620nm wave length employed. In order to visualize the three colors observed in the multiplex reaction (green, orange and red) a long band-pass filter originally designed to visualize Ethidium Bromide was utilized.

Discussion

Despite the economic, social and ecological importance of the introduction of GMO crops into the environment, following up their spread has remained an elusive goal. For example, when considering the crucial importance of accounting for GMO releases and their adoption in the US agroecological landscape, Marvier et al (2008) noted " The widespread planting of GE crops in the United States since 1996 represents a grand experiment… Unfortunately, this experiment cannot be analyzed because we lack well-documented maps depicting the varying prevalence of crops with specific GE traits each year." This situation has not changed since.

Perhaps the most important reason for the lack of geographical information of the spread of heritable materials from GMOs is the relative difficulty and the centralization inherent in the methods used to detect their presence or absence at a given time or place. Burdened by a complex labyrinth of commercial, legal and public debates over GMOs, current statements about their presence absence rely on commercial—and to a much lesser extent, academic—laboratories where experts using PCR amplifications and sequencing produce results which cost hundreds of US dollars for each determination. In other words, the complexity of current (PCR-based) methods of detection prevent non-experts from contributing to the knowledge about presence/absence of GMOs in specific locales, and with this it also precludes the finer and more useful goals of continuous monitoring or eventually mapping of these engineered life-forms.

Isothermal methods of DNA amplification provide a well recognized element in a strategy aiming towards the dual goal of reducing the cost of detection, while at the same time producing presence/absence results with a method that is robust and simple enough to be performed effectively and reliably by non-experts in remote field conditions (Urdea et al., 2006).

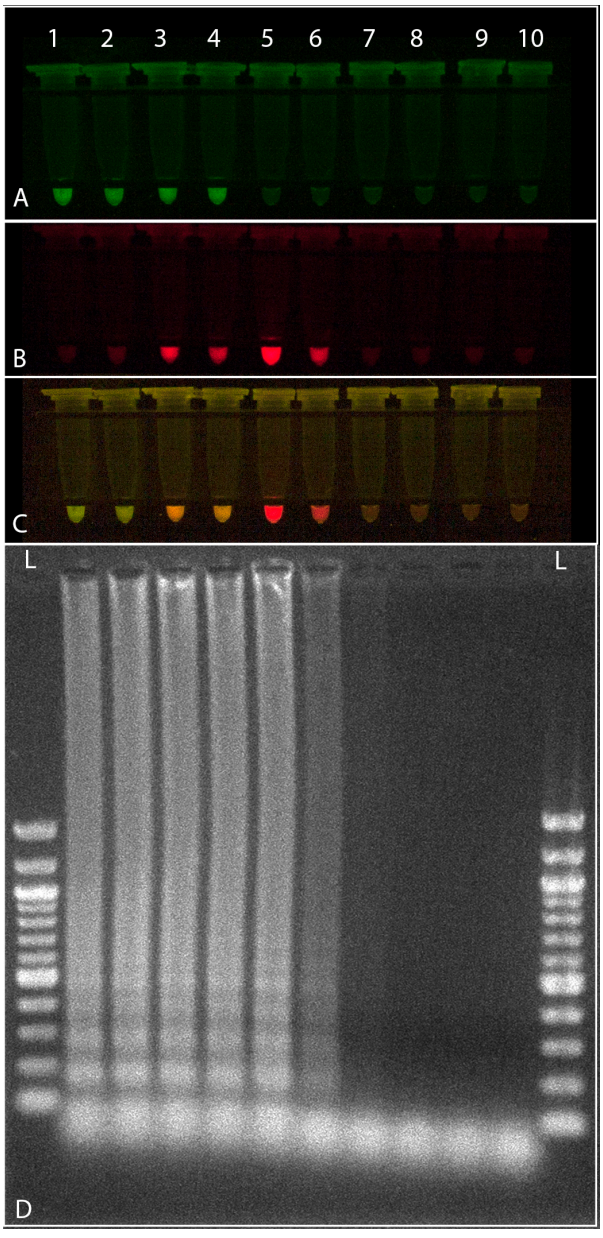
In particular, LAMP has demonstrated its benefits on the counts of both cost and robustness. This method, which obviates several delicate and costly aspects of PCR amplification has been shown to be effective in a variety of situations of medical and veterinary importance (Abdul-Ghani et al., 2012; Dukes et al., 2006). Here we have demonstrated the applicability of LAMP to the case of GMO detection. We have further shown that it is possible to utilize the LAMP method as a multiplex system for the simultaneous determination of presence/absence of at least two different DNA sequences relevant to GMO detection in the same sample.

Because our method allows also for the performance of the LAMP reaction on single-pollen grains, the LAMP multiplex method described here effectively determines the absence/presence of two independent markers in a single individual grain. Whether this capacity can extend to single-cell reactions remains to be determined, since pollen grains typically contain three genomes resulting from meiosis and recombination associated with the plant's microsporocite.

Our method allows for the application of multiplex LAMP not only to minute amounts of biological materials, but also to do so without the need to perform, strictly speaking, an extraction or purification of the DNA in the sample (Bektaş & Chapela 2014, 2015). Added to previously-described qualities of low-toxicity, extremely low background of false-positives, and robust application even in years-old samples (Bektaş & Chapela 2014, 2015), this method has the characteristics needed for a low-cost, field-able method which can be applied in the field, in relative independence from laboratory and other infrastructure previously required by PCR-based methods.

Figure 5.1

L: Ladder, 1-2: Maize DNA, 3-4 Triple-Stacked GM Corn, 5-6 GM Soy EPSPS, 7-8 Cedar DNA, 9-10 Water Control
Panel A: Tubes observed with a filter specific to fluorescein
Panel B: Tubes observed with a filter specific to TexasRed
Panel C: Tubes observed with a long-band pass EtBr filterPanel D: Gel electrophoresis with lanes corresponding to tubes.



Chapter 6
Conclusion: Widespread Adaptation of Field-based detection of transgenic sequences: current possibilities and scenarios for future development

The material presented in this dissertation establishes the foundation for our final goal: a portable for the detection of DNA moving through the environment.

We have adapted the decade-old Loop Mediated Isothermal Amplification method to be able to distinguish between DNA from pollen grains of various species with the final reporting of the results being via fluorescence. We have also begun investigating the lower and upper threshold limits for pollen grains of this method. As a laboratory we are interested in the detection and mapping of all microbial life in media that can sampled in a high-throughput manner (such as air and water). But we are specifically interested in following the movement of transgenic DNA both because of its social implications but more significantly because its foreign and easily distinguishable characteristics.

Transgenic constructs provide the perfect sequences to follow the movement of DNA in and out of individuals, species and ecosystems. Having been formed in the lab, outside of classical evolutionary dynamics, they are distinct chimeras easily distinguishable from other DNA sequences, and therefore potentially easily followed through ecosystems and species.

As we have outlined in Chapter 4, the device in question must possess characteristics as defined by the World Health Organization's *ASSURED* acronym: *A*ffordable by those interested/impacted; *S*ensitive, with few false negative results; *S*pecific, with few false positive results; *U*ser friendly, requiring only minimal training to obtain results; *R*apid; *E*quipment-free; with methods; and with results *D*elivered as directly as possible to those who need it (Urdea, 2006) (Box 4.1). More specifically, and for the purposes of mapping transgenes it must be able to detect single particles through DNA sequence specificity, multiplexed to detect multiple sequences, be low-cost and free of toxics and applicable to remote field-based situations. Table 6.1 outlines how our methods accomplish these goals.

Our constraints in developing the process of amplifying DNA from airborne pollen grains have been directly informed by our overarching desire to provide counter-power to the biology of corporations, the defense industry and the academic research institutions at their service. We are not only interested in democratizing the tools for biological research but also in developing novel tools more appropriate for a democratic research agenda and conceiving of the social relationships forged within this agenda (Harris, 1998).

At this juncture, where the biochemistry of a high-throughput, field-based detection of microbial DNA has been mostly developed, there are still a few remaining

technical considerations until a pilot study of detection devices can be released into the world.

Remaining Technical Developments

Delivery of Reagents

The components of the LAMP reaction need to be prepared in a manner that would allow for the travel and storage in ambient temperatures around the world. In conjunction, the reconstitution of the reagents must be straightforward and result in a homogenous mixture.

Lyophilization of molecular biology reagents is the industry standard for transfer and long-term storage (Park and Kim, 2000). Oligonucleotides used for DNA amplification are routinely prepared and shipped to the end-user in lyophilized form, as are the buffers that are dissolved from these solid states.

Unquestionably the most sensitive component of any biochemical reaction is the enzyme(s) utilized. These proteins with their delicate tertiary forms are especially susceptible to denaturation brought about by high temperatures. Many teams of researchers and commercial investigators prepare enzymes in lyophilized form (Klatser et al., 1998; Siegmund et al., 2005). The second generation of DNA amplification enzymes (Hot Start DNA polymerases,) have also been prepared to be relatively more thermostable by the addition of a variety of aptamers (Lin and Jayasena, 1997).

Since the reagents must be delivered to a sample already prepared to be amplified (see below) one proposition is that the necessary quantity and concentration of lyophilized reagents are delivered encapsulated in a degradable vesicle, deposited within the necessary volume of water, agitated and centrifuged to form the final mastermix.

Sample Preparation and Delivery

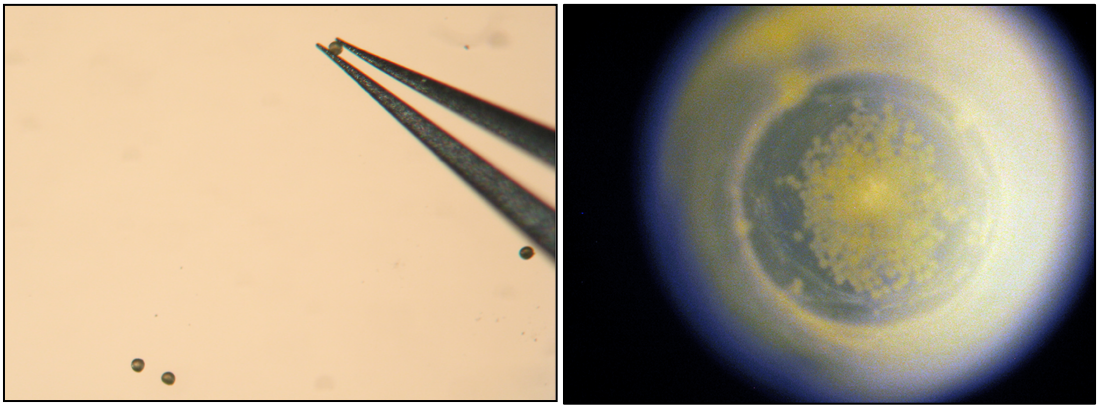


Image 6.1 Picking pollen grains and depositing them into a reaction tube

Until now we have been working with pollen grains picked underneath a dissecting microscope or extracted from honey samples via centrifugation and filtration. The lysis and permeabilization of the pollen cell wall is achieved through the action of a strong base (0.1M NaOH) and heating (17min at 90°C). Following this step is the essential neutralization of the sample prep in order to bring the pH to a value amenable to the activity of the amplifying enzyme Bst 2.0.

The protocol currently in place still relies on laboratory access and the separate multiple stages of lysing and neutralizing. This must be modified to become field applicable perhaps through a timed release of reagents as determined by thermal properties of encapsulating vesicles, similar to the delivery of LAMP reagents.

In addition to the preparation of the sample for amplification, the sampling unit and delivery must also be carefully considered in regards to the research question and investigation at hand. Microorganisms such as bacteria and viruses can be directly amplified without a lysis step (Aymerich et al., 2003; Rodríguez et al., 1994), but pollen and spores with their resilient cell walls require pre-treatment.

Primer Design Schema

As described from the onset, throughout the development of this novel method we have strived to make ourselves, i.e. those in the laboratory, as obsolete as possible. Although we have taken all the steps towards this there remains one essential aspect which requires a trained and skilled professional: the design of oligonucleotides (aka oligos) specific to the inquiry.

The process of oligo design is ardous and time-consuming. It is as complicated as dictated by evolutionary dynamics. As such, designing oligos to distinguish between closely related species such as *Zea mays* (corn) and *Zea mays ssp. mexicana* (teosinte) is much more difficult than those part of different genera. Publically accessible sequence data in databases such as GenBank which can be analyzed with free tools such as BLAST (Altschul et al., 1990) greatly expedite the process of primer design. In the absence of already established sequence data, sequencing must be conducted before any of the following steps such as alignment and primer design can take place.

The design of oligos for LAMP must be confirmed outside of the software process by way of in-lab testing across a variety of species, especially sympatric ones. This is a necessary step for the design of any amplification procedure but especially crucial for LAMP, which can utilize between four and six separate oligos with six to 8 distinct annealing regions. Even further, since LAMP is an isothermal reaction the annealing temperatures of all of the priming sites must be in close range for the reaction of be robust and specific.

In our experience designing LAMP primers for a variety of pollen grains we have found that the post design laboratory validation across species is of outmost importance

before any field application can take place (Bektaş and Chapela, 2014). This not only insures the robustness of the primers for detecting the sequence in question but also eliminates, to the best of ones ability, any possibility of false-negatives.

So then, how to reconcile the tension between our desire to have an autonomous network of people detecting DNA of their choice and the necessity of having a specialized group of people to design the oligos necessary? The only feasible solution is to have dedicated nodes at the service of the network where requisitions can be made, primers designed and tested (Figure 2.1B). Ideally, there would be multiple laboratories undertaking this endeavor not only for the sake of decentralization but also because different laboratories will have access to different sample material to test the robustness of the primers.

Pilot Detection Network

Our lab has been sampling airborne pollen for the past 10 years in an effort to better understand how these potentially transgenic DNA containing microbes move through the air. While working in Mexico we found ourselves headed in a new yet completely complementary direction for our work, which would ultimately provide the perfect venue to put our methods to test.

In 2011, the European Court of Justice ruled that honey was to be brought up under the GM regulatory framework of the European Union. Previously ignored, the presence of pollen grains in honey was now recognized as plant genetic material and therefore non-approved GM events to be contained to below 0.1% in honey. Suddenly the 148,000 ton honey trade into Europe was brought under scrutiny and exporters from around the world scrambled to come into compliance with the situation.

The effects of the new regulation were particularly drastic for the medium-to-small-scale honey producers in Mexico, which provides 11% of Europe's honey imports. When it comes to honey labeled *organic*, Mexico is the third largest exporter in the world with 85% of its production destined to Europe (Thompson, 2012). Due to our team's prior expertise on GMO detection in Mexico a number of honey investigators, cooperatives, producers and distributors asked us to assist with a cost-effective and decentralized testing procedure.

Honey and Pollen

A sample of honey or pollen basket form a hive contains a specific profile of the ecosystem from which honeybees forage for pollen and nectar (Marchand, 2013). Furthermore the low level of humidity in honey provides an ideal storage and preservation medium of microorganisms (Olaitan et al., 2007). Beyond these properties, the culture of beekeeping with its care of observation, ideological emphasis on the purity of honey (placing it in opposition to transgenic contamination (Lamping, 2012))

and the great number of enthusiasts around the world (Crane, 2013) provide an ideal social group to undertake a pilot study of field-based, autonomous detection of airborne DNA.

Our collaborations in the world of honey are diverse but the principal ones stretch form Chiapas, Mexico to Aragon, Spain and end in Wuppertal, Germany. The plethora of samples we have begun to obtain from these sources are also varied in that some of the samples originate from low GM zones, others from areas with extensive GM cultivation and further others have already been tested for presence/absence of transgenic material.

ECOSUR - Chiapas, Mexico

As the news of EU regulation came in, we were immediately contacted by the foremost apiary research team in Southern Mexico, based out of El Colegio de la Frontera Sur (ECOSUR) in San Cristobal de Las Casas, Chiapas. Our colleagues there, Rémy Vandame and Eric Vides were embarking upon fieldwork to establish honeybee foraging distances to crops that have GM varieties, namely soy and corn (Vides & Vandame, 2012). But above and beyond this research imperative they also communicated the near panic felt by the beekeepers whose main export market, Europe, was suddenly becoming jeopardized—or at the very least, more costly—due to the added expenses of DNA testing and certification. In visits to ECOSUR and a yearlong sabbatical by Dr. Vandame at our lab we have cemented this relationship and identified an initial group of beekeepers in the state of Yucatan to partake in our pilot study for field level detection of transgenic pollen within honey. The Yucatan Peninsula produces approximately 40% of Mexico's honey with 95% of it destined for export markets (Echazarreta et al., 1997). It is also where the majority of transgenic crops, soy, have been grown in Mexico and thus this peninsula presents the most urgent location to undertake this work. In addition, most of the producers are organized into cooperatives that want the ability to conduct testing on their own.

Aragon, Spain

In the European context, where GM crops are relatively more regulated than all other regions of the world, one area stands out in contrast by allowing the widespread cultivation of transgenic corn. Spain has allowed the MON810 transgenic corn to be planted since 1998 with a total of 100,000 hectares being cultivated today. The great majority is planted in the state of Aragon.

Aragon also is home to 7% of Spain's honey production. Our longtime associate Rosa Binimelis brought to our attention that the new regulations on the sale of honey within the confines of the European Union were naturally creating a stir with the honey producers of Aragon. In an initial field visit to probe the possibility of working in the

region our lab was fortunate enough to meet Joaquin Arqué Villar, a local organic farmer in the process of completing his Agricultural Engineering degree.

This collaboration has already resulted in the collection of more than 60 samples of honey in the transgenic corn-growing region of Aragon. Together with Mr. Villar we have conceived of a thorough workflow in order to test the applicability and robustness of our method against others that have become industry standards (Figure 6.1). This is to act as model for the whole of the pilot study, helping us fine tune our methodology for distinct environments around the world, whether that be the small scale agricultural system of the Mexican *milpa* or the much more industrial Spanish context.

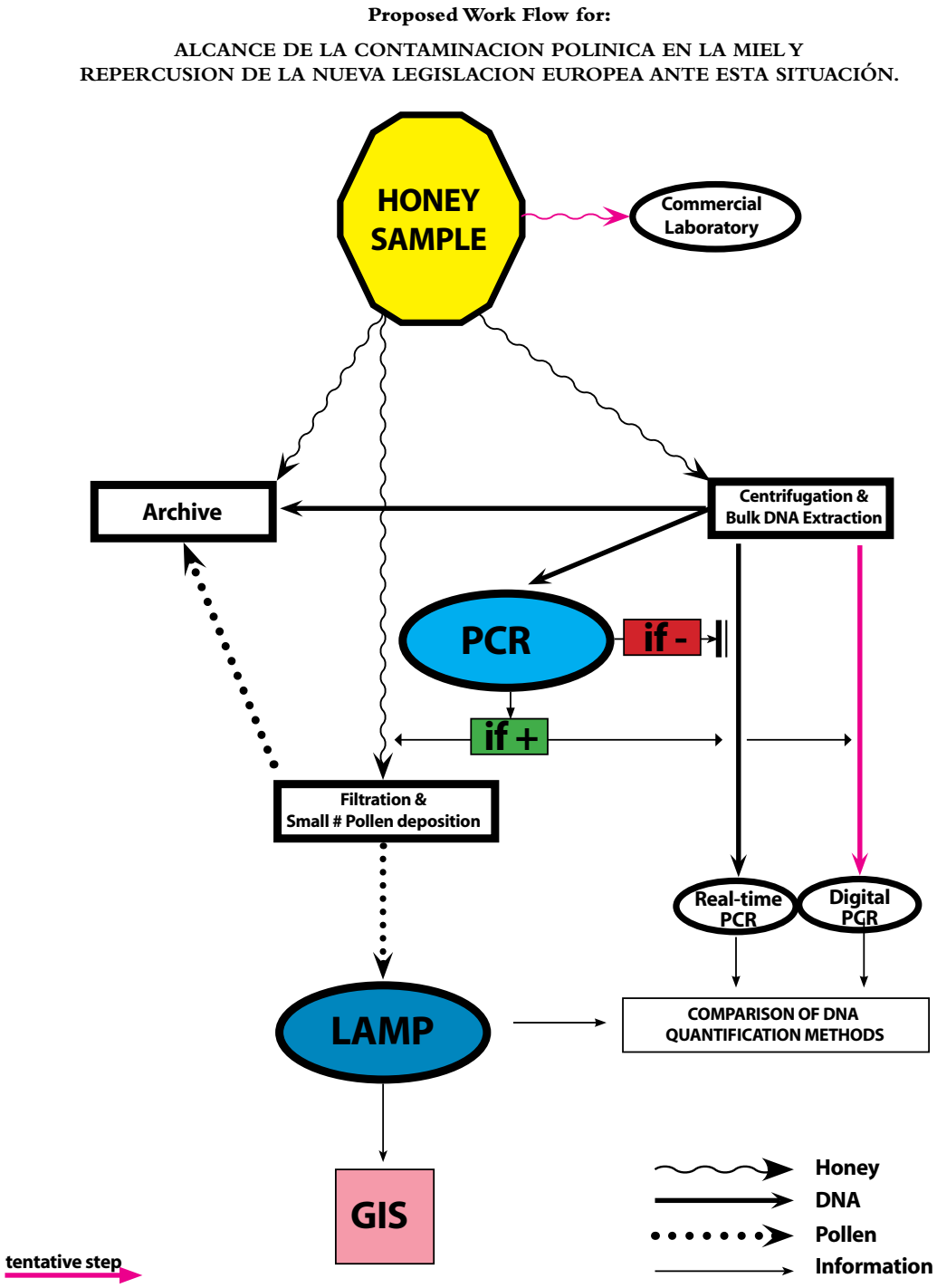


Figure 6.1 *Workflow for collaboration between the Chapela Lab of Microbial Ecology (University of California Berkeley) and Joaquin Arqué Villar (University of Zaragoza)*

GEPA, The Fair Trade Company – Wuppertal, Germany

It is crucial to position our efforts on both ends of the production-to-distribution chain in order to monitor the movement of DNA from ecosystems to consumers and beyond. An essential collaboration at the distribution side of the apiary chain is the one we have been forging with the German fair trade company, GEPA, the largest of its kind in Europe.

A challenge in making ecosystem-wide claims from honey samples has to do with the nature of industrial honey production. In most cases large-scale producers of honey look to maximize their profits by renting out hives for "ecosystem services," namely pollination for insect-pollinated crops such as a variety of fruits including apples and berries as well as almonds, pumpkins and squash. This dual-profit from honey sales and pollination-services implies that the hives are not kept stationary and are transported great distances where pollination is in demand by the market. The resulting honey is not representative of an ecosystem but of a market dynamic[3].

This is not the case when it comes to what is labeled "fair-trade honey" since fair-trade implies small-scale and locally produced products from less developed countries. This political and economic imperative makes GEPA one of the largest repositories of honey whose pollen is representative of a particular ecosystem. Over the course of three different visits we have obtained over 300 samples from honey producing communities in Mexico, Argentina, Uruguay, Bolivia, Chile and Ethiopia. Some of these samples have already been tested by commercial laboratories and classified as containing transgenic material or not. This presents a welcome opportunity to compare and contrast our methods with those that are the industry standard. The fact that the transgenic status of these samples are known, and thus are not blind samples as with the case with the Mexican or Spanish ones, give us ideal conditions to test the applicability and reliability of our methods.

For GEPA, fair-trade also signifies maintaining a close relationship with the producers of the products that they distribute. Similar to our connections in Southern Mexico, those who sell their honey to GEPA have also expressed an interest in participating in the pilot program of detecting transgenic pollen in honey.

[3] Although our emphasis is on ecosystem-level investigations one can easily imagine a fruitful project looking at the distribution of pollen grains and/or transgenic DNA sequences which illuminate how flowering plants, bees and the "pollination services" interact by way of the honey market.

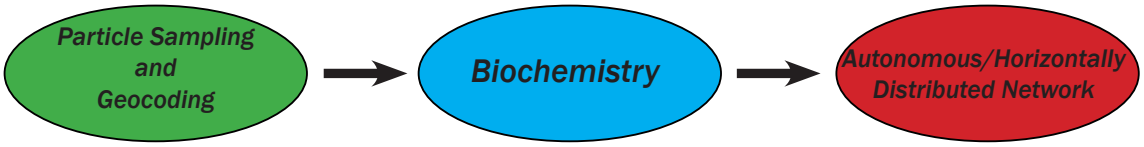
Beyond Point-sample Testing, Towards an *Ecoscope*

As indicated at the onset of this dissertation, the invention and development of the microscope changed the whole of biological sciences. At that epoch the emphasis of biological investigation was geared towards understanding and describing forms of life. Today, the minutiae of microbes and cells are still barely understood whether it be about development, heredity or intracellular communication. But the emphasis of biology as a science has shifted dramatically as more and more researchers are probing questions related to global distributions, shifts and flows of said microbes. Globalization has had an impact on the sciences as it has on all other domains of human life.

If this is the new focus, and if our political project is to rescue the investigation of biology from the confines of institutions where hegemonic power is concentrated then what would an instrument facilitating this in the realm of microbial ecology look like? Before answering this question it is useful to briefly look at what the current proposal for the global investigation of the distribution of DNA and of microbes looks like.

With the rapid proliferation of sequencing technology, and especially with the improved efficiency bringing down the price of per base-pair sequencing, many research units have invested in this technology. The result has been the global collection of soil and water samples for the purposes of wholesale sequencing. The resulting information is then flowed into a host of bioinformatics software to parse out Open Reading Frames and other sections of DNA deemed significant. Individuals and their distributions are inferred form millions of base pairs by algorithms (Caporaso et al., 2012). The major assumption in this equation is that our understanding of organization of the genome is certain, but if this is not true, which mounting evidence seems to point to (Latham, 2011) , then what is the value of the data? The rebuttal is that at least the whole genome sequence is present for future bioinformatics, but what if genomes are not static entities and have a certain dynamism to their nature (Rayner, 1997)?

Our proposal, based on the work presented here, takes a vastly different approach to the problem. Instead of high-throughput sequencing all of an organism's DNA we propose to detect in a high-throughput manner organisms carrying a particular known stretch of DNA. As the microscope has changed the playing field we aim to have an impact on the field of DNA ecology by introducing *The Ecoscope:* a portable device capable of sampling, amplifying and analyzing DNA in a **high-throughput** fashion. The biochemical reactions described in the preceding pages sit squarely in the center of this device with the power of providing a positive or negative result from a single pollen grain as indicated by a fluorescent signal.



On the upstream side of the biochemistry, the ecoscope will have the capability to sample the environment via flowing media, such as air or water. Microscopic particles will be collected and fixed upon a medium[4] while being assigned a geolocation. This will allow for the high-throughput sampling and analysis of each particle. Such capacity will bring about the potential to exponentially increase the current resolution of mapping microbial life within ecosystems.

This potential can only be realized by a radical reconceptualization of the social relationships involved in biological research. No single research unit could possibly undertake the amount of work necessary to make dynamic maps, which provide and update information on the distribution of microbes and their associated DNA fragments. Moreover, beyond pragmatics, part of our goal from the onset is to brake such centralization for the purposes of disseminating the power and knowledge which arises from understanding how life-forms function.

For this end we are developing the aspects of the Ecoscope downstream form the biochemistry to be a decentralized network of collaborative mapmakers. The Ecoscope, as a device, will have the capacity to transfer the data acquired, GPS coordinates and presence/absence data for the DNA sequence queried to a collaborative GIS system. Beyond providing a repository of maps, this network will also facilitate real collaboration by being a portal for disseminating research questions pursued by nodes in the network. In addition it will facilitate the cross-checking of results between different nodes, not only by mapping of the same regions by different people but also by sending samples to each other to have the results validated. This network will also facilitate the design of primers by identifying the most pressing needs for the users of the Ecoscope.

With the steps illustrated here, our methods promise to bring the capacity of DNA detection to beekeepers and assist them in addressing their ecological and commercial concerns. Further development and the eventual creation of the Ecoscope promises to redefine the field of microbial ecology by providing a much higher resolution of mapping the presence/absence of DNA sequences in air and water. On top of the technical innovations presented here, the social and political priorities our team has will ensure that the research and their results will have consequences that bring into question and challenge the power structures at play within the science of biology today.

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[4] The material for this process has already been determined but is being considered for patent qualification for the purpose of attracting established production companies to engage in the development of an inexpensive and mass-distributed device.

Method Properties	Desired Qualities	Grounds	Possible Approaches	Approach Taken in this Dissertation	Accomplished?
Single-Particle Detection	Ideally, Yes/No output from a reaction, even when only one gene- copy exists in it	Yes/No output allows for simple detection, interpretation and digitization of data	LAMP, PCR and others	LAMP	Yes, 100%
DNA-Sequence Specificity	Ideally, single-nucleotide polymorphisms detected, and distinguished with low rates of false positives.	Allows for maximum flexibility, e.g. identification of individual varieties, insert evolution, etc. Low rate of false positives is crucial in socially-important subjects, such as GMO-contamination	LAMP, PCR and others	LAMP	Yes, but not to single-nucleotide limit. Clearly different genomes, such as GMO-loaded ones, can be easily and specifically distinguished. We achieved extremely low rates of false positives.
Multiplexing (More than one detection per reaction)	As many Yes/No returns on individual, independent "queries" for each single reaction. Queries represented by individual target sequences.	Multiplexing reduces the time and reagents required to obtain more answers to probing "queries" for DNA sequences. On the downside, multiplexing creates more complex detection challenges.	LAMP, PCR and others	LAMP	Yes, on principle. We demonstrated multiplex reactions with 2 queries, resulting in tri-color output. The complexity of biochemical reactions involved, however, raise questions about how far multiplexing can be taken.

Isothermal operation	Reactions that can be performed without the need for accurate and fast thermocycling.	The time, cost, expertise, maintenance, and operating inputs of thermocycling may be the most important hurdle keeping DNA detection out of distributive architectures of users.	NASBA, HDA, RCA, SDA, LAMP	LAMP	Yes. We have demonstrated the operability of an isothermal method, even in conditions free of electricity, using solar-heating.
Toxic-Free Processing	Reactions for DNA amplification, and visualization free of chemicals, radiation, or other toxicants.	If our methods are to be used in a horizontal, distributive manner, we should expect direct exposure to reagents and materials by humans and environments out of our control.	Several approaches in the last decade, but none had been completely compliant	LAMP, avoiding DNA-binding dyes, avoiding UV radiation, avoiding heavy metals in electronics	Yes, in principle. Our methods can be developed to complete compliance, although for convenience we performed them still using electronics, UV and plastics.
Electricity-Free Process	From sampling to detection in an environment independent of stable electricity sources.	Independence from reliable sources of regulated electricity opens the possibility of using our methods in truly remote locations.	Several; there are some precedents such as heating through chemical reactions	Pilot process using solar heating	Yes, albeit in a cumbersome manner. We have not developed a streamlined method to operate free of electricity.
Refrigeration-Free Process	Storage and operation of the process autonomous from reliable "cold chain"	Applicability in remote field locations, possibility of using surface post, stability of stored reagents are all necessary requirements for a method for remote field use in a distributive-horizontal network architecture	Lyophilization, glycerol storage, solid-state chemistry	Lyophilization	No. We have not devoted enough effort to this goal.

Low Cost	As low a cost-per-reaction as possible	Cost remains the paramount limitation to distributive-horizontal and autonomous detection.	Avoid costly materials, economies of scale	LAMP, Low-cost visualization	Yes. Current cost per sample using PCR is in the hundreds of dollars, we currently perform reactions at an estimated USD$0.80/sample
Tolerance to environmental "noise"	Extraction-free, purification-free preparation	Reducing cost, as well as accommodating a wide variety of environmental contexts for each sample	Trial-and-error over the last decade	LAMP	Yes. Given small sample sizes, our method has been totally insensitive to environmental "contaminants". We have an extraction- and purification-free method
Automation: *Sampling*	Sizing through filtering and light scattering. Particle deposition alongside geocoding and other metadata (i.e. meteorological) and sample preparations	Establishing the precise location of particles alongside information relating to their origin.			
Automation: *Reaction*	Reagent delivery and heating	Hands free processing of the reaction			

Automation: ***Detection***	Light sources, optics and a sensor to read fluorescence (possibly utilizing sizing module)	Rapid detection of the results of the end-point reaction			
Automation: ***Reporting***	Software capable of conjoining the data collected during sampling with reaction results	Prepare data for GIS			
Automation: ***Data Processing***	A networked GIS system to visualize results on a map and to facilitate collaboration	Collaborative analysis of results			

Table 6.1 *Desired qualities of the Ecoscope*

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Glossary

35s promoter (p35s) and nos terminator (nos-t): Two of the most common DNA elements used in creating transgenic plants. Outside of the laboratory and outside of the transgenic context p35s is found in the cauliflower mosaic virus while nos-t is found in *Agrobacterium tumefasciens.* These two DNA elements have also become somewhat of a standard in screening for transgenes, although not all transgenic plants contain them.

Bioinformatics: The convergence of DNA sequence data and computing intensive statistics to deduce DNA patterns found in cells. A natural extension of regarding DNA as code, this field has witnessed a huge growth in the last ten years together with the high amount of sequence data produced.

Chimeric DNA: A DNA molecule artificially created in the laboratory as a collage of other DNA molecules from a variety of organisms that are usually not reproductively compatible.

DNA Sequencing: The determination of the order of adenine, guanine, cytosine and thymine in a deoxyribonucleic acid molecule. The cost of producing this data has been brought down considerably in the past decade from $1000 to less than 10 cents per raw megabase-pair of DNA sequence[1] thanks to new molecular reactions where templates are immobilized, synthetic DNA adapters are used and automated detection of base-pairs are perfected. These advances have superseded the standard Sanger reaction and paved the way for a plethora of dedicated sequencing facilities in large universities, corporations and private laboratories.

Enzyme-linked immunosorbent assay (ELISA): A common test to detect antigens (protein or peptide chains) in a particular sample through the binding of a specific antibody linked to the activation of an enzyme. The enzyme drives a biochemical reaction usually resulting in a readily visible color change.

Horizontal Gene Transfer (HGT): The movement of DNA between organisms, which are sexually incompatible and belong to distant phylogenetic domains. Almost always facilitated by microbes, HGT can happen in the laboratory or in the open field. In a strict sense, trasngenesis is the human appropriation of HGT.

Human Genome Project: The effort at the end of the 20th century to determine the order of all of the nucleic acids from a single human being. Often labeled a race, there

[1] The National Human Genome Institute (NHGRI) regularly tracks cost of sequencing.

were two research units working on this project, one a publicly funded initiative, the Human Genome Project, and the other Celera Genomics. At the end the two teams made their final completion announcement together in June of 2000 with Prime Minister Tony Blair and President Bill Clinton present at the ceremony. President Clinton went as far as to famously declare that "Today, we are learning the language in which God created life."

Ice-Minus Bacteria: Developed by Advanced Genetic Sciences (an off-campus start-up by a group of Berkeley professors) to combat frost-driven destruction of crops, this transgenic bacterium was sprayed on to a California strawberry field in 1987 marking the first intentional environmental release of a transgenic organism.

Landrace & Introgression: A landrace is a persistent population of a species that has co-evolved with its specific ecosystem, including human cultivation, over evolutionary time. Introgression refers to the sudden incorporation and stabilization of hereditary material into such a population.

Lyophilization: The process of removing water by way of sublimation under a deep-cold, low-pressure chamber.

Mon810: One of the most widely cultivated varieties of transgenic corn in the world. Owned by Monsanto this variety produces the Bt toxin, ostensibly against the pest insect known as corn borer. It is also the principal transgenic crop approved for cultivation in the European Union.

Phylogeny: The evolutionary relationships between life forms, evidenced by their features such as morphology or DNA sequence.

Teosinte: A complex of genera and species that form the evolutionary ancestor of corn. In Mesoamerica teosintes are sympatric with corn and continue to exchange hereditary material with the crop. Aragon, Spain, is the only part of the world where it is currently regarded as an aggressive weed.

Transgenic Organism: A life form, created outside of its natural context, in a laboratory via the crude introduction of DNA or other heritable material from one or more life forms with which the original life form would not be sexually compatible.

Type I (false positive) and Type II errors (false negative): Type I errors derive from rejecting a null hypothesis which is true. Type II errors occur when a false null hypothesis is not rejected. In other words, and concerning the detection of a particular DNA fragment from a sample, a false positive is when the detection results in a positive

despite the absence of the DNA sequence in the sample. A false negative is the failure to detect the presence of a DNA fragment within the limits of detection of the reaction.