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In vitro plant regeneration from petiole-derived callus and characterization of inter Simple Sequence Repeat (ISSR) markers of Amorphophallus Hewittii Alderw. (ARACEAE).

Douglas Charles Bertelsen

IN VITRO PLANT REGENERATION FROM PETIOLE-DERIVED CALLUS AND CHARACTERIZATION OF INTER SIMPLE SEQUENCE REPEAT (ISSR) MARKERS OF AMORPHOPHALLUS HEWITTII ALDERW. (ARACEAE)

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IN VITRO PLANT REGENERATION FROM PETIOLE-DERIVED CALLUS AND CHARACTERIZATION OF INTER SIMPLE SEQUENCE REPEAT (ISSR) MARKERS OF AMORPHOPHALLUS HEWITTII ALDERW. (ARACEAE)

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University of Nebraska, 2007

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The genus *Amorphophallus* contains many species that are sought after by collectors. A large proportion of these species do not reproduce quickly enough to satisfy the demand. Consequently, the establishment of micropropagation methods to increase the supply is desirable. Though several protocols for tissue culture do exist, these focus on species of agricultural importance that naturally reproduce vegetatively. This research demonstrated the successful *in vitro* reproduction of *Amorphophallus hewittii* Alderw., a species that is not known to reproduce vegetatively. The protocol avoided destroying the parent plant through use of petiole material. It concluded with direct transfer of shoots to soil, eliminating the *in vitro* rooting stage and simplifying the acclimation of plants from culture. Molecular markers based upon Inter Simple Sequence Repeat (ISSR) were developed to determine genetic stability during the tissue culture process. These markers are useful in a wide variety of investigations. Sufficient loci were found to use this technique in this species. This study reports the first successful *in vitro* protocol for this species and the first ISSR markers for a non-agricultural *Amorphophallus* species.

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Introduction

In vitro culture is a method capable of rapidly creating many cloned offspring plants from a single plant. This form of artificial multiplication is ideal for plants that mature slowly or do not reproduce large numbers of offspring. Several members of the genus *Amorphophallus* demonstrate these traits. One such species is *Amorphophallus hewitii* Alderw.; this plant flowers infrequently and is not known to reproduce vegetatively.

The first objective of this investigation is to develop an efficient protocol to multiply *A. hewittii* by applying principles from existing *Amorphophallus* spp.

Many studies have shows that a number of plants arising from *in vitro* culture are not genetically identical to the source tissue. Molecular markers have proven useful as only a tool to measure genetic stability through culture, and can be applied to a wide range of biological studies.

The second objective of this investigation is to develop a set of molecular markers to visualize and quantify the mutations arising during *in vitro* culture.

The Genus *Amorphophallus*

Natural History and Growth Habit

The genus *Amorphophallus* consists of at least 170 species of aroids (members of the family Araceae) occurring primarily in secondary growth or disturbed paleotropical

forests across Africa, Asia and Australia (Hetterscheid and Ittenbach 1996). The species of this genus are perennial, sending up a single leaf during the growing season and resting via underground corms or rhizomes during less favorable conditions. Tropical members in *Amorphophallus* may retain a leaf longer than a year. This single leaf is superficially similar to, and occasionally as massive as, a small tree; with the "trunk" composed of an herbaceous petiole and the "branches and leaves" formed by branching rachises and leaflets (Figure 1.1).

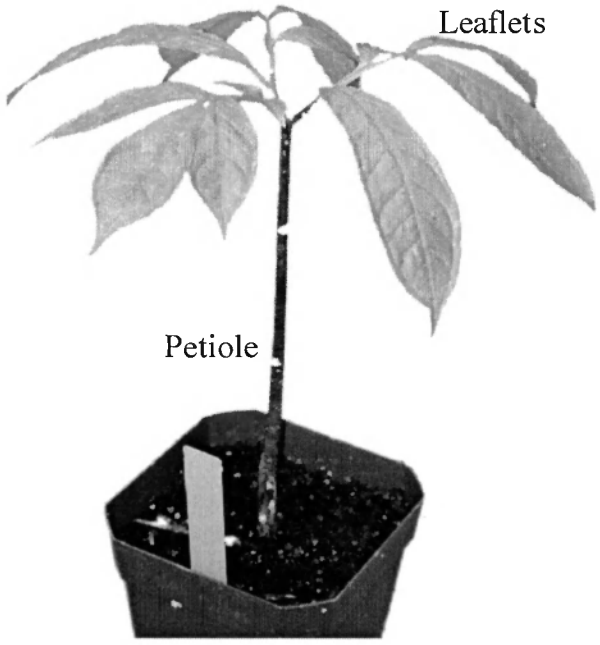


Figure 1.1. A young *Amorphophallus hewittii* plant. Note that all vegetative structures visible form a single leaf with the vertical petiole and spreading leaflets. The entire leaf will senesce before or during production of a new leaf.

When the plant reaches maturity, a process that in some species may take over a decade, it produces an inflorescence subtended by a leafy spathe that appears similar to those of peace lilies (*Spathiphyllum spp.)* or calla lilies (*Zantedeschia spp.),* two aroids common in the horticultural trade. Separate male and female flowers are arranged along

the central spadix (Figure 1.2). These inflorescences range from four centimeters in *A. pusillius* to a gargantuan three meters as in the case of *A. titanum*. The inflorescences are protogynous, thus preventing self-fertilization; several cases of pollination by the same plant and between clones have been mostly unsuccessful. Plants may only bloom every two to three years and the stigmas are only briefly receptive (a few hours to a day) to pollination by flies and carrion beetles attracted to the flower by its odor (Hetterscheid and Ittenbach 1996). Most floral odors in the family are likened to rotting meat and explain why *Amorphophallus* spp. are often referred to as "corpse flowers." Successful pollination is followed by maturation of the seed in a brightly-colored fleshy fruit; birds are considered to be the main dispersal agent of seeds (Hetterscheid and Ittenbach 1996).

Figure 1.2. *Amorphophallus linearis* inflorescence. The male and female flowers are situated around the spadix, which is subtended by a leafy spathe. These inflorescences are very short-lived if not pollinated.

Research on the genus *Amorphophallus* has primarily focused on systematics, floral physiology, and agricultural uses of its species. Much of the information regarding culturing and natural history has been anecdotal, collected by amateur naturalists and horticulturists interested in the genus. These observations were presented with a brief description and plates of plants commonly found in culture by Hetterscheid and Ittenbach in the International Aroid Society's journal *Aroideana* (1996).

Demand for *Amorphophallus* species

Commercial Uses

Two members of the genus, *A. konjac* and *A. paeoniifolius,* have received more attention than the rest due to economic importance. Both are valued as food sources (Misra and Swamy 1999). Powdered corms of *A. konjac* are medicinally useful in treating constipation (Loening-Baucke et al. 2004), improving weight loss, and lowering cholesterol (Walsh et al. 1984). *A. konjac* is also used in the commercial production of mannose (Cescutti et al. 2002). *A. albus* corms are harvested in China for glucomannan, but on a limited basis due to its three year cultivation period before harvest (Hu et al. 2006). Agricultural crops of *Amorphophallus* are typically grown in monoculture or are interspersed with other crops such as coconut. Plants are placed in pits fertilized with manure and, upon maturing, the large corms are dug up with spades and crowbars (Misra et al 2002).

Horticultural Interests

Perhaps the best recognized species is *A. titanum* because of its massive size and the interest generated at universities and botanical gardens worldwide when it blooms.

While the flowering *A. titanum* is not as rare as it once was, due to the wide distribution of seeds and the use of tissue culture, large crowds still gather to observe this botanical curiosity. The remaining species are typically only of interest to researchers and individuals seeking to grow them. Though not as severe as tulipmania in 17th century Holland, collectors are willing to pay several hundred dollars for a larger or more rare plant. These prices can often be attributed to the novelty of a newly described species or difficulty in sustaining enough plants to meet demand due to long reproduction time or lack of vegetative reproduction.

Reproductive Methods

Within the genus, wide variability exists in reproductive behavior. While some species become sexually mature in the relatively short time of 2-4 years, most take much longer. Vegetative reproduction often takes place in the form of offsets from the parent corm, rhizomes or bulbils produced on the leaf (Figure 1.3). Offsets typically take one to several seasons to mature and may be directly attached to the parent corm (Figure 1.3) or arise from short rhizomes (Hetterscheid and Ittenbach 1996). Plants may produce 17 or more offsets in one growing season (D. Bertelsen, personal observation) in the case of *A. paeoniifolius*. Bulbils (Figure 1.3) are masses of undifferentiated tissue found on the leaf, either along the rachises (epiphyllar) or at the point the petiole meets the leaf blade (intercalary). These are usually distributed within a short distance of the mother plant when the leaf senesces; clumps of plants are found in species where this form of reproduction occurs (Hetterscheid and Ittenbach 1996).

Figure 1.3. Vegetative reproduction in *Amorphophallus*. Left: Three cormels (a) forming on the underground corm of *A. haematospadix*. Right: *A. bulbifer* with bulbil forming at the junction of the rachises.

Within this genus, species such as *A. konjac* (offsets), *A. paeoniifolius* (offsets) and *A. bulbifer* (bulbils) are known to reproduce vegetatively, while many others such as *A. titanum* do so only when the plant is disturbed or damaged (Hetterscheid and Ittenbach, 1996). This adaptation allows the plant to resume growth in the event of loss of the main growth point. Naturally, species reproducing freely are typically more available from horticultural sources and less expensive than those species where vegetative reproduction is absent or slow.

Means of Traditional Commercial Propagation

As previously mentioned, many species of *Amorphophallus* will at least occasionally reproduce vegetatively. Relying solely on vegetative reproduction may result in few or no plants, depending on the species. Those that reproduce large numbers of offspring are widely established in culture (*A. bulbifer, A. konjac,* and *A.*

paeoniifolius); while many species of horticultural interest do reproduce vegetatively, they often do so at a rate insufficient to meet demand (Hetterscheid and Ittenbach, 1996).

Large-scale multiplication has been reported in the case of *A. paeoniifolius* (Misra et al. 2002). The traditional method employed involves sectioning larger corms into multiple smaller pieces that are planted in a similar fashion to seed potatoes. Dormant meristems located on the surface of the corm are activated and one or more shoots grow from the piece. This method has several disadvantages when considering plants less common in cultivation. Up to twenty percent of the crop from the previous year provides starting material for the new crop. In the case of plants where only a few corms are available, removing corm material reduces the biomass available to produce the next leaf; this leads to either a smaller or non-existent original plant. The loss of a large mother plant can be a costly sacrifice if the entire corm is used. Harvesting only a portion of the original corm provides a method to preserve the original plant but may reduce the value of the plant and extend the time before sufficient corm mass exists for flowering. Wounds resulting from partial harvest of the corm may allow pathogen entry into a tissue that is susceptible to necrosis and secondary pathogens (Hetterscheid and Ittenbach, 1996). Although many species may be successfully multiplied *in vivo*, pathogen contamination of stocks has been cited as a factor reducing plant quality (Gandawijaja et al., 1983) when *Amorphophallus* is considered an agricultural crop. *In vitro* propagation of *Amorphophallus* spp. is also desirable because of slow maturation rates, the lack of vegetative reproduction in many species (Hetterscheid and Ittenbach, 1996) and the absence of viable pollen due to rare flowering events, in addition to the previously

mentioned occurrence of pathogens.

Current Research Involving *Amorphophallus*

Hetterscheid and Ittenbach are currently working on major revisions of both the Asian and African groups of *Amorphophallus*. Research groups in India, China and Japan are studying agricultural aspects including use of molecular markers to study cultivar relatedness in *A. konjac* and *A. paeoniifolius*. The *in vitro* protocol and/or molecular tools described in later chapters could be applied to either study.

Tissue Culture of *Amorphophallus*

Published Protocols

Development of *in vitro* culture for *Amorphophallus* species has been relatively limited. Commercial applications are often cited as a motivation for *in vitro* culture for both *A. konjac* and *A. paeoniifolius* (Cescutti et al., 2002). To date, only a few published *in vitro* protocols exist for non-agricultural species, including *A. titanum* (Kohlenbach and Becht, 1988. Though suggested as a useful method to preserve this endangered plant species, the authors did not report if the protocol was suitable for a reintroduction program or merely to meet commercial demand. Protocols for *A. albus*, *A. kachinensis* and *A. yunnanensis* (Zhuang and Zhuo, 1987) and a second protocol for *A. albus* have been reported (Hu et al. 2006). Inorganic components of media followed that of Murishige and Skoog (1962) for all the protocols except for *A. titanum* and *A. konjac* (Kohlenbach and Becht 1988). These two protocols employed several modified Nitsch media as previously described (Pierik 1976; Geier 1986).

Explant Tissue Sources

All of these species for which tissue culture protocols have been established, with the exception of *A. titanum,* are known to reproduce vegetatively, whether by offsets, rhizomes or bulbils (Hetterscheid and Ittenbach; 1996) and may lend themselves to tissue culture by utilizing explants that would normally be amenable to organogenesis. Two protocols for *A. paeoniifolius* report utilizing explants composed of a lateral bud and small amount of corm tissue (Irawati et al. 1986, Nyman et al. 1987). The activation of these dormant meristems would most closely mirror natural plant responses to loss of the primary growth point. Interior corm tissue was used in one protocol for *A. konjac* (Asokan et al. 1984) with a brief callus formation and shoot differentiation occurring in eight to nine weeks after initial culture. A viable alternative to tissue culture using corm material, preserving both the corm size of the parent plant and reducing the chances for soil-borne pathogens is the leaf itself. Protocols for *A. konjac* (Zhuang and Zhou 1987; Kohlenbach and Becht 1988; Hu et al. 2005), *A. titanum* (Kohlenbach and Becht, 1988), *A. albus* (Zhuang and Zhou 1987; Hu et al. 2006), *A. kachinensis* and *A. yunnunensis* (Zhuang and Zhou 1987) call for propagation via petiole segments through formation of dedifferentiated callus followed by shoot organogenesis. One trial using the leaf lamina as explant material (Kohlenbach and Becht 1988) provided no viable cultures on the media in the study.

Parameters of Tissue Culture

Premise and Stages of Tissue Culture

In vitro propagation of plants or portions of their structure, has developed theoretically and practically over the last century. The guiding principle in tissue culture is multiplication of a selected plant under controlled conditions. These include high nutrient levels and axenic conditions to eliminate bacteria, fungi or other pathogens that might compete or damage the plant. Under these conditions plants can rapidly multiply. The total number of plants may double within a few weeks or months.

Plants are organisms that produce all new structures (roots, shoots, and modified types of both) via meristems. While plant cells may grow and divide apart from these meristems, the new cells are functionally and morphologically defined by chemical signals from surrounding cells and tissues (Scheres 2001). Cells in a meristematic region are characterized as totipotent, i.e., able to differentiate into any type of plant cell; not unlike stem cells found in animals. Meristems were used in early tissue culture to grow roots isolated from any photosynthetic tissue (White 1934) and commonly serve as a source of new plants in the traditional method of reproducing a plant by taking cuttings.

Micropropagation, the process of multiplying plants *in vitro* with smaller sections of tissue than *in vivo* propagation, in many plant species is as simple as taking microcuttings of a sterilized plant and repeatedly dividing them. The new plant grows from meristems developed in the same manner meristems would form in a plant found in nature. This method is relatively simple and can produce many thousands of plants per year as long as the species of interest naturally produces many shoot meristems.

When considering other species lacking this rapid production of meristems, a different course of development may be employed through tissue culture. In the absence of meristems suitable for producing new plants, cells may be chemically dedifferentiated; under the right conditions, subsequently divided cells lack functional or structural specialization and will continue dividing into undifferentiated cells until they are chemically triggered to differentiate into one of the many types of specialized plant cells. Micropropagation via organogenesis relies on this developmental pathway as a source of meristems. All except one *Amorphophallus* spp. protocol employ this shoot organogenesis from undifferentiated material.

The process of tissue culture has traditionally been divided into three stages (Murishige 1974). Stage I is the disinfection and initial introduction of the explants to *in vitro* culture. Stage II, the multiplication stage, includes any steps necessary to induce the plant to reproduce vegetatively. This may include activation of axillary meristems, somatic embryogenesis, callus induction or adventitious shoot development. Stage III prepares the plant for transplant from culture and may include rooting and/or hardening. Since each of these stages involves promoting a different physiological response, it is common that each stage involves a particular set of conditions including nutrition and environmental parameters differing from the other stages.

Protocol Development

Development of a suitable protocol for *in vitro* culture of a plant may be time-consuming and costly (Kyte and Kleyn 1996). Optimizing a protocol fully would entail studies regarding the source of plant material, individual optimization of dozens of

inorganic and organic compounds, media preparation, selection of a strategy by which to multiply the plants, and adjustment of a range of environmental conditions including temperature, lighting levels and photoperiod. Fortunately, many of these parameters have a limited impact on *in vitro* production and do not need extensive study unless difficulties arise.

Explant response is largely determined by concentrations of growth regulators when other factors are favorable for the plant; of secondary importance are tissue source, energy source, photoperiod and vitamins (Torres 1989). Initial studies on protocol development tend to focus on growth regulator identity and concentration. Often, two or three growth regulators are combined in a range of concentrations to determine optimal formulation as judged by desired response (Torres 1989). This process of growth regulator optimization may need to be repeated for each stage in the culture process.

In vitro culture occurs in some form of closed system in order to prevent entry of contaminants. Even if filtered air and circulating liquid-phase media are utilized, plants must still rely on the media for all mineral nutrients. While it is possible to optimize individual levels of each inorganic compound, many protocols call for one of several dozen common formulations derived for a particular class of plants (McCown and Lloyd 1981), initiating a physiological response (Fujita et al. 1981) or as a generalized medium for many differing plants (Murishige and Skoog 1962). As stated before, most *Amorphophallus* spp. protocols use some modification of Murashige and Skoog (MS) media, which was developed to culture tobacco (*Nicotiana tabacum)* callus (Murishige and Skoog 1962) and has since been used in hundreds of other genera.

Tissue Culture Media Contents

The typical constituents in tissue culture media can be categorized as gelling agents, macronutrients, micronutrients, carbohydrates, vitamins, and growth regulators. Media preparation involves several factors that may affect culture results including pH adjustment, sterilization method, and duration of sterilization (Torres 1989).

Gelling Agents

Gelling agents provide a mechanical support and may provide osmotic control for the plant. Common gelling agents are agarose, gellan gum, and other less frequently used compounds such as cornstarch and gelatin. Agarose, a polysaccharide derived from several species of red algae, has been the gelling agent of choice for many decades but is relatively opaque, may contain impurities that may interfere with cultures, and is expensive to use on a commercial basis (Torres 1989). Many substitute gelling agents offer improved clarity, purity or decreased cost. The identity of gelling agent has been linked with culture response (Van Ark et al. 1991).

Inorganic Nutrients

Macronutrients and micronutrients are defined as inorganic ions acquired from soil and functional *in planta.* Macronutrients include ions needed in relatively large quantities for normal plant growth and development and include calcium, magnesium, nitrogen, phosphorus, potassium and sulfur. Micronutrients are ions needed in smaller proportion than macronutrients; among them are boron, copper, iron, manganese, molybdenum, and zinc. These are usually added in chelated form, reducing the rate of

precipitation (Torres 1989).

Carbon and Energy Source

Cell proliferation may be accelerated in culture as compared to similar tissues found *in vivo* and are subjected to conditions under which autotrophy, generation of all required photoassimilate (sugars or starches) from light and carbon dioxide, will not provide the plant with enough energy and carbon molecules for optimum growth. Even in the rare occurrence where a culture is capable of autotrophy, supplementation of carbon dioxide may be required for long-term maintenance (Rogers et al. 1987). In order to compensate for lower light and a lower available carbon dioxide rates, addition of a carbohydrate, most commonly sucrose, allows the plant to operate as a mixotrophic (partially dependent on photosynthesis) or heterotrophic organism. Under the latter state, plants or the organs under culture do not need to produce any chlorophyll and may be cultured in complete darkness for extended periods of time. Both carbohydrate concentration and identity are important when developing protocols (Khuri and Moorby 1994).

Vitamins

Vitamins, organic compounds that act as cofactors and regulators in plants, are required for normal growth and development (Ohira et al. 1976). They are commonly added to tissue culture media, as they may be a growth-limiting factor in cell growth (Torres 1989). Vitamins commonly added include nicotinic acid, thiamine, pyridoxine, and ascorbic acid. Vitamins are typically produced endogenously by plants and may not be required except in the case of low-density cell cultures (Ohira et al. 1976). Most of

the common media formulations call for them (Murishige and Skoog 1962; Gamborg et al. 1968; Anderson 1975).

Plant Growth Regulators

The group of media constituents requiring the most fine-tuning is the plant growth regulators (PGRs). Though new categories of growth regulators including methyl jasmonate, jasmonic acid and brassinosteriods are being characterized (Ravnikar et al 1993; Clouse and Sasse 1998), four major groups are recognized as significant in tissue culture of a wide range of species: auxins, cytokinins, gibberellins, and abscisic acid (Table 1.1). These categories are largely based upon their biological activity; cellular response is determined by cell type and may be on opposite ends of a response spectrum for different cells in the same plant. Relative activity within the groups varies by chemical identity and responses are dependent not only upon concentration of the individual PGR but also upon the ratios in which they are present and the species or even cultivar of interest.

Auxins generally stimulate cell growth, callus formation and root initiation. Indole-3-acetic acid (IAA) is the only naturally occurring auxin, but several others including 2,4-dichlorophenoxyacetic acid (2,4-D), 1-naphtaleneacetic acid (NAA), and 1H-indole-3-butryic acid (IBA) find use in tissue culture.

Cytokinins are a class of PGRs that stimulate cell division and shoot initiation. Naturally occurring compounds among this group are N-(2-furanylmethyl)-1H-purine-6-amine (kinetin) and 6-(4-hydroxy-3-methyl-trans-2-butenylamino)purine (zeatin). In addition to those naturally occurring, synthetic cytokinins such as 6-benzylamino purine

(BAP) or 6-γ-γ-dimethylaminopurine (2iP) have been used with success in many tissue culture protocols.

The last two groups of PGRs are less commonly used, but may be required for certain protocols. Gibberellins (commonly GA_3) and abscisic acid (ABA) enhance stem elongation in dwarfed plants and enhance shoot proliferation, respectively.

Table 1.1. Overview of plant growth regulators. Major classes, their common physiological action on plants and several examples used in tissue culture are listed

Plant Growth Regulators

Class	Predominant action (s)	Examples
Auxins	Stimulate callus, cell elongation, root initiation	IAA, 2,4-D, IBA
Cytokinins	Cell division, shoot initiation, promote axillary meristems	Kinetin, 2iP, BAP, zeatin
Gibberellins	Seed germination, shoot elongation, callus growth	GA_3
Abscisic acid	Promote or supress callus growth, increase shoot multiplication	Abscisic acid

Undefined Additives

Occasional additions to growth media include undefined organic additives such as coconut milk (liquid endosperm) and other plant and animal extracts such as casein

hydrolysate. These may provide traces of PGRs or other compounds such as amino acids. Activated charcoal may be included in the media to absorb toxic phenolic exudates or promote root development by blocking light. Most protocols do not employ these unless necessary (Torres 1989). Casein hydrolysate was reported as a component for *A. titanum* media (Kohlenbach and Becht 1986) but its necessity is dubious.

Source of explants for *in vitro* experimentation has involved sterilized seeds, embryos, leaf lamina, stem sections, roots, storage organs, pollen grains and many other sources, the source being largely determined by availability, ease of surface sterilization, and purpose of the culture. Culture success can be dependent upon tissue source and age of tissue. As mentioned before, *in vitro* protocols for *Amorphophallus* spp. have focused on petiole sections and, to a lesser degree, corm material; these tissues being both predominant in a non-flowering specimen and lending themselves to sterilization.

Measure of Success in Tissue Culture

Benefits of Tissue Culture

Tissue culture offers several benefits over both wild-collection and traditional vegetative propagation. It is possible to introduce a wild plant into tissue culture by only removing a sample of tissue without disturbing the root system or injuring the corm. This may be the best option when considering a highly imperiled species. In cases of increased availability, a sample of wild plants may be moved to the site of culturing and used as stock plants. Seeds also provide an excellent source of tissue and include the added benefit of genetic variation. Methods such as meristem culture and thermotherapy

(increase of temperature to destroy pathogens) provide an avenue for eliminating pathogens such as fungi, bacteria and virii from wild-collected plants and have been used on other members of Araceae (Hartman 1972; Li et al. 2002) to provide clean stock plants.

Drawbacks of Tissue Culture

Tissue culture provides a method for rapid production of large numbers of pathogen-free plants; however, it is important to note that several drawbacks do exist. Those commonly encountered are temporary aberrations caused by culture conditions (Bednarek et al. 2007), loss of genetic diversity, and genetic mutations arising from culture (Larkin and Scowcroft 1981).

The conditions under which plants are cultured may have adverse effects upon plant morphology. Hyperhydricity (presence of excess fluid in cells or tissues), excess branching, or physiological changes in any number of tissues may arise in plants *in vitro*. These physiological responses may either revert after a short time out of culture or do not pass the traits on to progeny (Torres 1989).

Genetic Consequences of Tissue Culture

Ideally, plants generated through tissue culture would be exact genetic clones of the parent plant as no sexual reproduction has taken place. While consequent genetic uniformity may be valuable in many horticultural plants where near-simultaneous blooming or another desirable trait is to be maintained, conservation-based projects place value on capturing as much of the genetic variation and identity as possible (Allen 1994). Reproductive incompatibility may become a problem when all cultured plants are

reproductively incompatible (de Nettancourt 1997).

Somaclonal variation (SCV) was coined by Larkin and Scowcroft (1981) to describe mutations arising *in vitro.* This includes single nucleotide polymorphism and chromosomal mutations such as deletions, inversions, duplication, and translocation. The loss of genetic fidelity has been observed in a number of species including sugarcane where up to 6.93% of meristem-cultured plants showed polymorphism over 98 RAPD (random amplification of polymorphic DNA) loci (Zucchi et al. 2002). One study on *A. konjac* reports that root tip cells showed between 22 and 45 percent variation in chromosomal number from expected with all plants appearing phenotypically normal (Huang et al. 1995). Chromosomal variation was not reported for a control group not undergoing tissue culture; further molecular studies should be conducted before making a determination whether tissue cultured plants would be suitable for reintroduction as part of a conservation program.

Properly maintaining an *ex situ* population of plants is difficult because of unintentional selection and high costs associated with maintenance of a large number of individuals (Snyder et al. 1996). This difficulty coupled with the pervasive nature of tissue culture mutations (Phillips et al. 1994) bring into question the applicability of releasing plants produced through tissue culture as part of a conservation effort. Perhaps the best use of tissue culture conserving *Amorphophallus* would be to ease the demand for wild plants by producing specimens for sale. This approach was applied to the rare *Alocasia guttata var. imperialis* (Burnett 1984).

Detection of Genetic Mutations

History

Initially, plants undergoing tissue culture were screened for mutations via a physical observation of progeny. This is sufficient to ensure a plant looks normal and the traits of interest are present. The karyotyping of cells undergoing mitosis allows a superficial confirmation that no major chromosomal mutations have occurred. This method was utilized when characterizing genetic stability in *A. konjac*; this study also implied that observed phenotypic appearance was unreliable as a determinant of SCV (Huang et al. 1995).

Molecular Methods

With the advent of molecular techniques, it has become possible to observe a molecular make-up of the organism (genotype) as a phenotype. Many molecular markers are co-dominant; allowing the heterozygote to be scored. DNA fingerprinting converts DNA sequences into textual, numerical or graphical data and provides the best method by which to directly detect or estimate frequency of mutations. Rather than rely on a handful of characteristics validated during visual screening or the low resolving power of karyotyping, a set of fingerprints can be created where hundreds of characteristics can be quickly screened visually or electronically. Four methods of generating these data have been commonly used: RFLP (Restriction Fragment Length Polymorphism), AFLP (Amplification of Fragment Length Polymorphism), RAPD (Random Amplification of Polymorphic DNA), and ISSR (Inter Simple Sequence Repeat).

RFLP

RFLP creates fragments of DNA by action of restriction enzymes on genomic DNA. These enzymes recognize a particular sequence of DNA (a restriction site) and cut the strand of DNA within this sequence. Genetic differences between two organisms may be located within the restriction site causing the enzyme to not cleave the DNA or a new restriction site to be recognized; alternatively, different lengths of DNA between two restriction sites will result in different lengths of fragments. These fragments are then separated by size through electrophoresis, blotted, and allowed to hybridize to a probe molecule (typically radioactively labeled). Any changes to restriction sites or the length of DNA sequences between two sites will appear as loss, addition or shifting of bands. RFLP is procedurally simple and robust but is dependent upon large quantities of DNA of relatively high quality (fragment length) to produce good results. It requires little to no previous knowledge of the genome of interest. With the limited amount of DNA available in this study, and the drawbacks of radiolabeled probes (short shelf-life, cost, and disposal) this method is feasible but has serious drawbacks.

AFLP

AFLP uses PCR (Polymerase Chain Reaction) to amplify fragments produced by restriction enzyme digestion by addition of adapter sequences to the end of restriction fragments before amplification (Vos et al. 1995). Amplification products are then separated by electrophoresis. It offers similar data to RFLP but does not require such large quantities of DNA due to the amplification step. Nonetheless the method is time-consuming and technically demanding. Large numbers of loci are generated per experiment, but full resolution requires the use of a larger PAGE (PolyAcrylamide Gel

Electrophoresis) apparatus.

RAPD

RAPD provides genetic information by amplifying random areas of the genome using primers (9 or 10 bases long) of arbitrary sequences to initiate PCR amplification (Williams et al. 1990; Welsh and McClelland 1990). Again, visualization of amplified fragments is accomplished via electrophoresis. It requires no prior knowledge of the genome of interest but suffers from reproducibility because of non-specific binding (Jones et al. 1997).

ISSR

Inter simple sequence repeat (ISSR) is another PCR-based method that utilizes a primer of short repeat length, typically two to six bases per repeat and is anchored at one end by one or more nucleotides (Gupta et al. 1994; Zietkiewicz et al. 1994). These primers are designed to anneal to SSRs (Simple Sequence Repeats) found throughout the genome (Figure 1.4). Benefits of ISSR over other molecular marker systems include reproducibility (Jones et al. 1997), hypervariability, genome-wide distribution (McCouch et al. 2002), and ease of development, as they require no previous knowledge of genomic sequences for the organism of interest. Most of the bands produced by ISSR are dominant, i.e, there is no method to distinguish a homozygous and heterozygous individual. Standard PCR (polymerase chain reaction) is carried out with an annealing temperature at or slightly above the melting temperature of the primer. This prevents nonspecific annealing and improves reproducibility. Amplified regions are those that contain two of the repeats on opposite strands within amplification distance (typically

two kilobases or less). The amplified PCR products are then separated by electrophoresis. Due to the technical simplicity, low cost and high yield of loci, this method is the most suitable for this investigation.

Figure 1.4. Method by which DNA is amplified in ISSR. Primers with multiple repeats of a short sequence, (TCG)6 in this case, are anchored by one or two nucleotides (C in this example). Polymerization occurs in a 5` to 3` direction. Loci where two annealing sites are inverted and within a short distance (typically 2kb or less) will be amplified geometrically, while those lacking a second inverted site will only amplify arithmetically.

Genetic Variation in Tissue Culture

Detection of Variation

Genetic distance, a measurement of differences in DNA sequence, can be determined in organisms using molecular markers. Methods for estimating genetic distance find wide use in biology to determine how many differences exist between individuals, populations or species. In the case of examining tissue culture clones, the genetic distance becomes a metric of SCV between the parent and offspring plant. Mutations detected by ISSR markers includes deletion of the amplified area and/or at

least one of the flanking SSRs resulting in a complete loss of the amplified band and additions or deletions within the band which show up as a shift in band size. Chromosomal inversions, duplications and translocations may disrupt an amplified sequence but must be verified by other methods.

Drawbacks not withstanding, ISSR has detected SCV in cultured plants. Six out of 224 calli from cauliflower (*Brassica oleracea var. botrytis*) showed variation from the parent plant when compared with four primers generating 149 ISSR bands (Leroy et al. 2001). Four primers amplifying 51 ISSR bands detected two instances of SCV in potato (*Solanum tuberosum*) (Albani and Wilkinson 1998). Studies using both ISSR and one or more of the other tools in population or systematic studies show good agreement between marker systems (Nagaoka and Ogihara 1997; Lakshmanan et al. 2007).

Calculating Genetic Distance

When considering success of tissue culture with respect to genetic fidelity using molecular data, the Jaccard similarity coefficient (Equation 1.1) is a method of determining similarity between two plants (van Eeuwijk and Baril 2001). While typically used in circumstances involving plants that are not necessarily identical, the equation can be extended to comparisons of parent plant and vegetative offspring, whether produced by tissue culture or natural reproduction.

Loci from the ISSR are scored based upon reproducibility and distinctiveness. They should appear in independent amplifications and be distinct enough to distinguish the loci from those flanking it. Each amplified fragment of DNA from the offspring plant

is compared to the set of fragments in the parent plant. Those that match are included in the term n_{11}. Those bands not present in offspring and bands found in offspring but not the parent are designated n_{10} and n_{01}, respectively. Since molecular data of the parent plant represent the target in the case of tissue culture, n_{parent} is used to represent the data of the parent, the inverse of the equation is taken, number of dissimilar data is subtracted from the number of similar data instead of adding as in the Jaccard coefficient (Equation 1.2). This rearrangement of the equation causes distance to be linear as number of similar data decrease. In the event the offspring plant has more dissimilar data than similar, the similarity does become negative in value. Precision of the calculated distance increases as the number of loci are increases.

Equations 1.1 and 1.2. Left: Jaccard similarity equation. Right: Modified Jaccard similarity equation to linearize similarity and set parent loci as target similarity.

$$S_{Jaccard} = \frac{n_{11}}{n_{11} + n_{10} + n_{01}} \qquad S_{JaccardModfied} = \frac{n_{11} - n_{10} - n_{01}}{n_{parent}}$$

Closing Remarks

Based upon past *in vitro* responses of *Amorphophallus* spp., development of a protocol for *A. hewittii* should be both possible and straightforward. While fine-tuning of conditions is necessary for optimal response between species, a good foundation of

example protocols outline a general method to regenerate plants from petiole-derived callus.

Because of the ubiquitous nature of SSR repeats in eukaryotes and the technically simple process of amplifying ISSR fragments, it is expected that many loci will be generated by screening a range of repeat sequences. Of particular interest would be those repeats that consistently amplify multiple fragments independent of the anchoring nucleotide identity. Such a group would suggest frequent occurrence in the genome. The tools developed may prove useful in applications outside the field of tissue culture in *Amorphophallus*.

By combining the ability of ISSR markers to detect DNA mutations with samples of tissue from various stages of the protocol, it may be possible to implicate a particular stage with such mutations.

Chapter 2:

In vitro *plant regeneration of* Amorphophallus hewittii *from petiole-derived callus*

Introduction

A. hewittii is slightly smaller than *A. titanum*, with leaves reaching 3 m high and inflorescences 2 m high, as compared to 5 m high leaves and inflorescences reaching approximately 2.5 m, and, like *A. titanum*, lacks regular vegetative reproduction (Hetterscheid and Ittenbach, 1996). Like most *Amorphophallus* species, *A. hewittii* is a desirable plant for many aroid growers, garnering premium prices as seeds, bulbs, or plants. We have developed a comparatively simple protocol for the *in vitro* multiplication of *A. hewittii* from its abundant petiole tissue.

Methods & Materials

Plant Material

Ten *Amorphophallus hewittii* seeds were acquired from Malesiana Tropicals (Malaysia) and grown under greenhouse conditions at the University of Nebraska at Omaha. All seeds germinated and were allowed to grow out for two years. The solitary leaf of a one plant was allowed to fully develop and regenerate corm material via photosynthesis before collection of explants. The petiole was cut 1-2 cm above the junction of the petiole and corm to prevent damage to either the root system or growth point. This point was typically located below soil level and lacked chlorophyllous tissue.

Tissue Sterilization

Petioles were stripped of all leaflets, washed under tap water for 5 minutes and then sterilized with a combination of 50% (v/v) ethanol and distilled water for one minute directly followed by a 15 minute submersion and agitation in a 1% (w/v) sodium hypochlorite solution with 0.1% Tween 20 added as a surfactant.

Culture Conditions

A series of media were inoculated with petiole sections (0.2 – 0.5 cm long). Lengths varied in order to maintain a constant mass of tissue in each explant. The entire length of the petiole was utilized, except for short discontinuities where the petiole was divided to aid the sterilization procedure. The mass and relative petiole locations of all explants were recorded for future reference. All cultures and plants were kept on growing shelves with a 15 hour photoperiod provided by compact fluorescent lighting ($2.4x10^4$ lux) and temperatures ranging from 25°C to 30°C. Cultures were monitored for signs of cell division, differentiation and contamination. Any cultures showing signs of contamination were disposed of or rescued through re-sterilization of the explant. Uncontaminated cultures, regardless tissue or media, were sub-cultured onto similar media every four to six weeks. Cultures were subdivided into two to four cultures when they would no longer fit into the tube. The subdivided cultures were then placed on similar media or transferred to media used for shoot multiplication trials. Cultures on shoot multiplication media were still transferred every four to six weeks until shoot meristems formed and appeared to begin active growth (Figure 2.1). Cultures were then rinsed and placed in soil-free potting mix under light stand conditions as previously

described. A leaf sample was taken for related molecular studies after the leaf fully expanded.

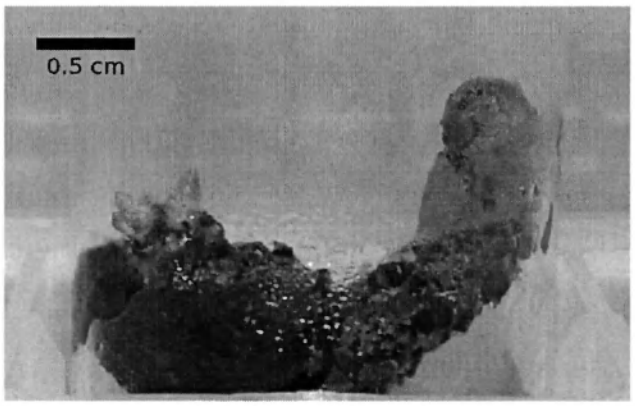


Figure 2.1. Early shoots on callus. One of the three pointed shoots on the left side subsequently elongated; the other two remained dormant. The dark color of the callus is due to a layer of dead cells. This coloration was typical of all calli.

Callus Induction

Twenty experimental media were prepared by amending MS media (Murashige and Skoog, 1962) with reduced ammonium nitrate (720 mg l^{-1}), ascorbic acid (100 mg l^{-1}), Gelrite (1.5 g l^{-1}), sucrose (3.0% w/v) and a range of NAA (0-13.5 μM) and kinetin (0-4.6 μM), pH 5.7. The best candidate media (NAA 13.5 μM and kinetin 2.3 μM) was selected by visual comparison of calli and used for further studies (Table 2.1).

Shoot Generation and Deflasking

The base media was modified for shoot generation by omitting the sucrose and increasing Gelrite to 2.0 g l^{-1} to account for weaker gel strength caused by absence of sucrose. Ten media were prepared with a range of NAA (0-0.05 μM) and kinetin (0-23.2 μM). The optimum medium (NAA 0 μM and kinetin 11.6 μM) was determined by

examining the number of shoots produced. Calli showing active shoot growth on this modified medium were transferred as previously mentioned. Root formation and hardening stages were unnecessary as plants were placed below soil level and allowed to grow naturally.

Table 2.1. Combinations of growth hormones tested. This table reflects the results of hormone concentrations on a sample of at least 12 explants per treatment. C denotes the media tested for callus production; S denotes shoot generation. Media with strong responses for callus, shoot or root formation are marked with a subscript 1, 2, or 3 respectively. The suggested media for callus and shoots are noted with a subscript O.

		[NAA] (μM)				
		0	0.05	0.54	5.4	13.5
[Kinetin] (μM)	0	C,S	C,S	C	C_1	-
	0.05	C,S	C,S_2	C	C_1	$C_{1,3}$
	0.46	C,S	C,S	C_1	$C_{1,3}$	$C_{1,3}$
	2.3	-	-	C_3	C_3	$C_{O,1,3}$
	4.6	S	S	C_1	C_1	C_1
	11.6	$S_{O,2}$	-	-	-	-
	23.2	S	-		-	-

Results and Discussion

Tissue Sterilization

Low rates of surface contamination were observed using the described protocol. Most contaminants began growth on the medium distal from the explant and were probably not directly from the tissue. Repeated subculturing sometimes resulted in an unidentified endogenous contaminant becoming obvious; no deleterious effects were observed in cultures this contaminant was present in. Culture rescue including surface sterilization and infiltration with the wide-spectrum biocide PPM (Plant Cell Technology) did not rid the cultures of contamination. Due to the late development of this contaminant with respect to the protocol, it is suggested that careful screening of parent plants and explant material of *A. hewittii* and the rest of the genus be performed before undertaking large-scale production through tissue culture. Identification of contaminant source (endogenous/exogenous) will direct any necessary modifications to the sterilization procedure.

Callus Induction

Petiole sections were initially filled with many air pockets that were crushed as cells divided and expanded. Resistance by the outer petiole layers caused the callus to expand outwardly from the cut surfaces of the explant. Cultures were initially off-white, but often developed a brown to black layer of dead cells at the surface. Under this layer were a thin chlorophyllous layer and the remainder of the off-white, and frequently purple, callus. This purple coloration was not observed in plants grown from seed under greenhouse conditions but may be chemically similar to pigments located in the petiole of

both the mother plant and clones.

The effectiveness of hormone complements in inducing callus was examined by varying NAA and kinetin in a modified MS medium. Media contained NAA concentrations from 0 μM to 13.4 μM and kinetin from 0 μM to 23.2 μM. Explants grown on the media exhibited responses along a continuum from no change to rapid callus growth. Organogenesis of roots was observed on several media. The roots appeared to be morphologically consistent with *in vivo* roots, complete with root hairs. The medium showing the most consistent callus proliferation (NAA 13.5 μM and kinetin 2.3 μM) was selected for further trials. The rate of mass accumulation was judged to be sufficient for culture purposes, though additional NAA may result in an increase in growth rate.

Callus growth rates on optimal medium were calculated on a percentage increase in fresh weight basis for each of the first three months. Fifty-eight separate calli, all from the same parent plant, were averaged; the calli increased an average of 238% (±87%) fresh weight per month. The increase in mass was lower during the first month in culture but stabilized thereafter. The large variation in fresh weight was due to several calli that increased in mass almost eightfold during a four-week period and calli that did not acclimate to culture quickly. Most published works do not cite rates of fresh weight accumulation; Irawati et al. measured callus diameter and found that their most effective medium approximately doubled the diameter of *A. paeoniifolius* calli every four weeks (1986). Assuming their calli were approximately spherical, this rate is comparable to the eightfold increase in fresh weight observed for *A. hewittii*.

Shoot Generation and Deflasking

No shoots were produced on the media optimized for callus proliferation. The medium with (NAA 0 μM and kinetin 11.6 μM) produced 1-9 shoots on most calli after 2-4 months; however, most of these shoots remained dormant while one or two shoots per callus elongated and produced leaves. Multiplication rates may be increased by decreasing callus size when transferring to shoot generation media or by dividing the calli after shoots have developed. Use of liquid media coupled with agitation (Asokan et al. 1984) or wounding of the main shoot (Hu et al. 2006) may prevent apical dominance and increase the number of shoots per callus and speed up shoot organogenesis. Cultures were washed, covered with potting mix and kept moist when one or more shoots began elongating. This method did not require the storage at 4°C that Hu et al. described as necessary to encourage sprouting (2006). Rooting and acclimation were accomplished naturally as the shoot elongated and broke the soil surface. One callus was kept under sterile conditions to observe plant development; it subsequently grew normal-appearing roots from the base of the shoots. Similar root development is evident in cultures that have grown under light stand conditions as seen in Figure 2.2. Details of the callus/shoot system are shown in Figure 2.3.

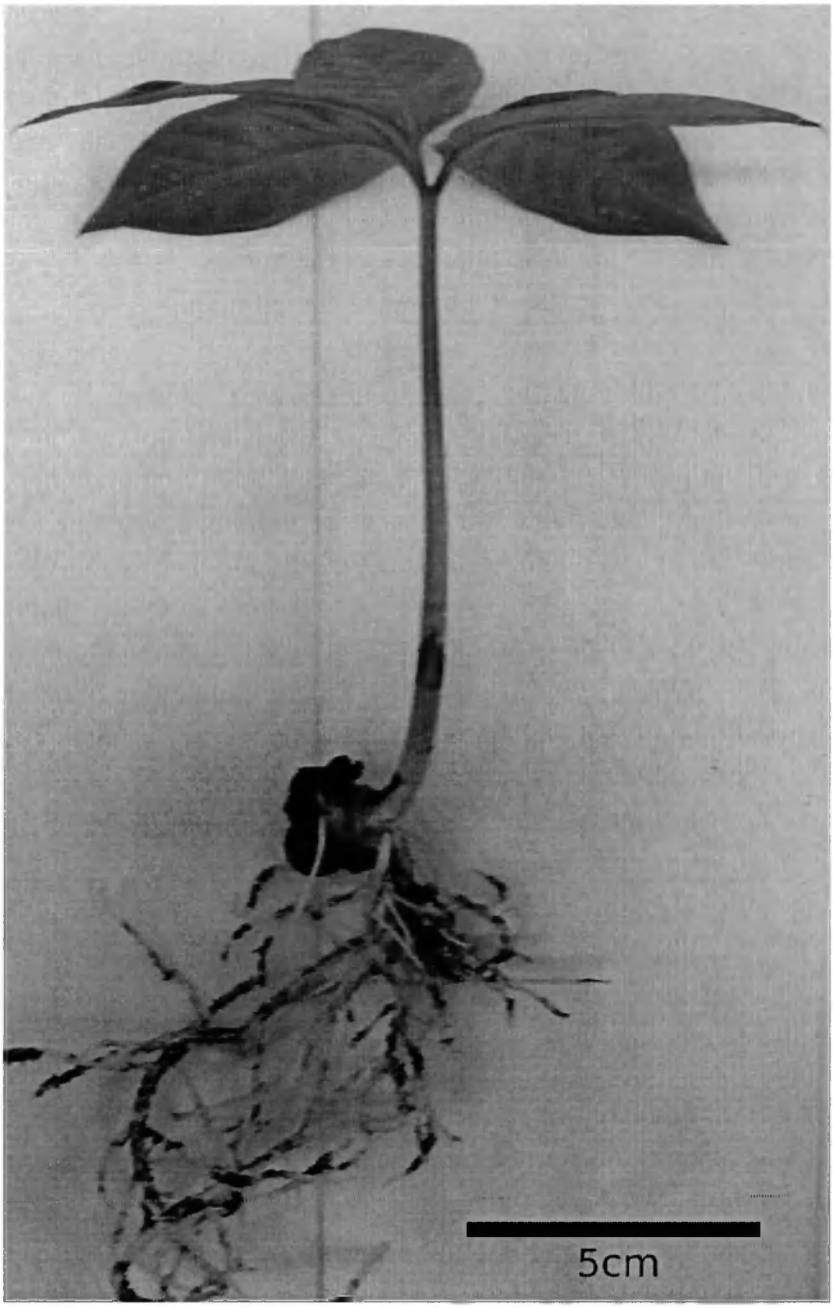


Figure 2.2. Fully developed *A. hewittii* six months after deflasking. Normal plant morphology is visible including the leaflets, petiole, corm and roots. The parent callus is visible as the dark tissue adjacent to the newly formed corm.

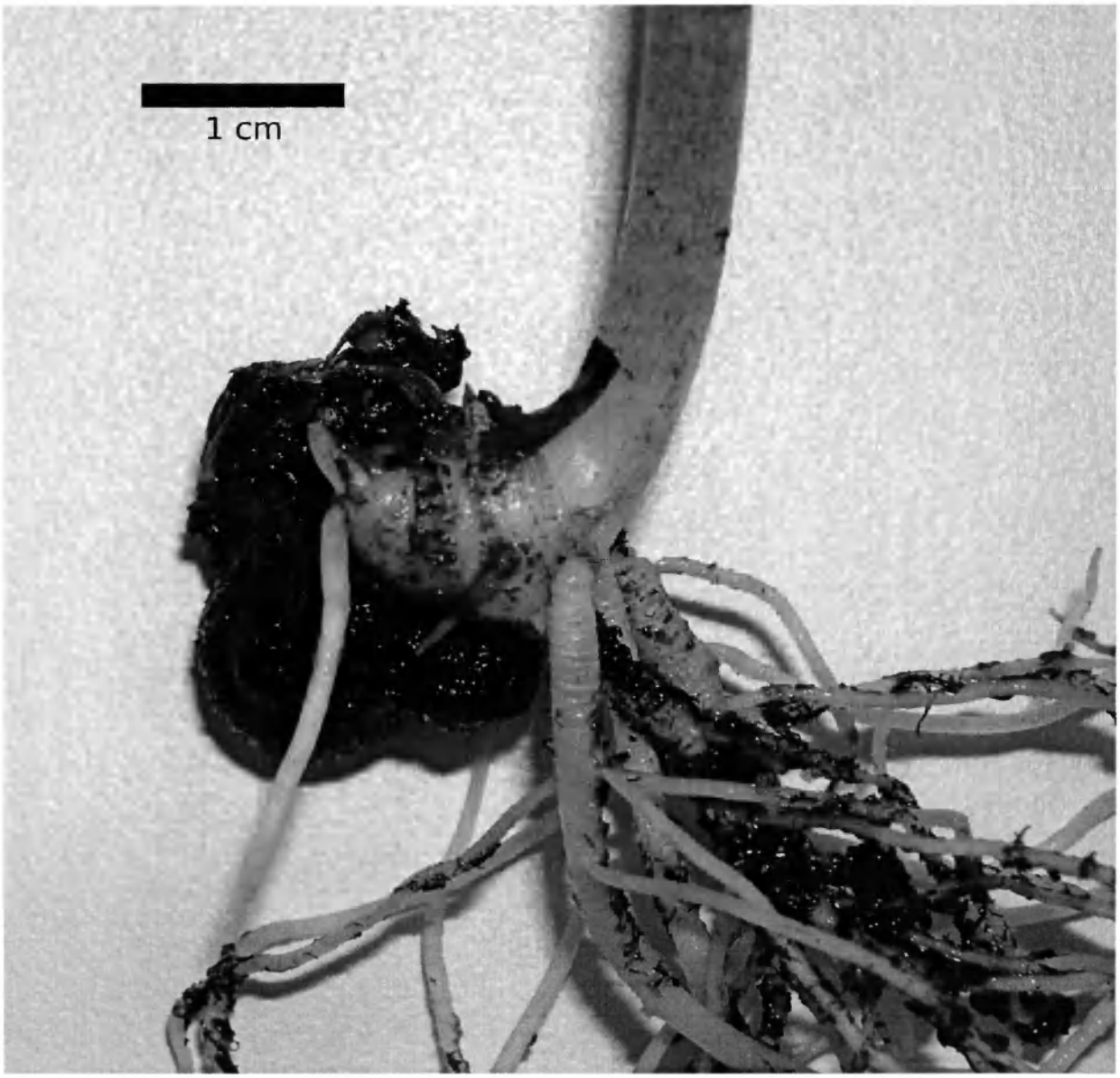


Figure 2.3. Detail of callus and corm six months after deflasking. The dark parent callus is still attached to the lighter corm tissue (produced after transfer to soil) by a bridge of live tissue. The leaf seen is the second to develop since deflasking; older roots may be seen closer to the parent callus while newer roots emerge closer to the petiole.

Conclusions

To our knowledge, this is the first reported *in vitro* propagation of *Amorphophallus hewittii*, and the first demonstration of successful transfer of elongating shoots along with the callus, directly to soil without either extended time in culture resulting in cormel formation (Irawati et al., 1986) or *in vitro* root development (Asokan et al., 1984; Nyman et al., 1987; Kohlenbach and Becht, 1988).

The relative simplicity (two media) and quick turnaround time (3-6 months) of this protocol compared to the protocols utilizing petioles as explant materials (Kohlenbach and Becht, 1988) also suggests that a more efficient method for culturing *A. titanum* and *A. konjac* may exist. Hu et al. (2005) used a similar two-step protocol with petiole sections to make histological observations of *A. konjac*, but did not document any root formation or transfer from *in vitro* culture.

Chapter 3:

Characterization of ISSR Markers for Amorphophallus hewittii

Introduction

ISSR was chosen over other methods due to its technical simplicity, reproducibility, and the ubiquitous nature of the loci in eukaryotes. It does not require multiple steps, radio-labeled probes or specialized equipment. The overall process of generating ISSR fingerprints has changed little since the method's proposal (Zietkiewicz et al. 1994; Gupta et al. 1994). Optimization of the protocol involves obtaining a quality genomic DNA sample, determining primers that provide multiple, scorable bands when visualized, and maximizing reproducibility by selecting the optimum annealing temperature for PCR. This optimization ensures the data provide an accurate reflection of the organism's genetic fingerprint.

DNA Extraction

Genomic DNA for PCR must be separated from cellular contents such as membranes and proteins that interfere with DNA amplification. Plant tissue offers additional difficulties when DNA must be extracted for molecular work. Typical plant cells are surrounded by a cellulosic cell wall. Even young tissues where this wall has not thickened must be mechanically disrupted before proceeding with the isolation of the DNA. Cell membranes and proteins are disrupted in a buffer solution by detergents and chaotropic substances, respectively. Organic solvents such as phenol, chloroform and iso-amyl alcohol are added to the aqueous phase to further denature proteins and

precipitate them. Centrifugation of the mixture results in separation of water-soluble compounds, a layer of cell debris including cell walls and proteins, and an organic phase that has absorbed several plant pigments including chlorophyll. Adding cold ethyl alcohol and gently agitating the solution accomplishes precipitation of the nucleic acids. The remaining pellet is then washed of other impurities with solutions in which DNA is not soluble. Addition of molecular-grade water or buffer solution then dissolves the DNA for PCR work.

By measuring the ratio of absorbances at 260 and 280 nm, rapid quantification and qualification of DNA may be performed. Nucleic acids strongly absorb light with wavelength of 260. Contaminants cause background absorbance at 280 nm to increase which lowers the absorbance ratio. "Clean" DNA usually has a ratio above 1.6 or greater. The concentration of DNA in a sample may be roughly calculated by measuring the absorbance at 260 nm.

Initial Screening of ISSR Primers

Primer selection for ISSR is often informed by successful amplification in a related species. At the time primers were selected for this study, no reports of ISSR in *Amorphophallus* could be located. In order to best screen for primers, all unanchored and single-nucleotide anchored dinucleotide repeats with the repeat combinations AC, AG, and CT were screened (Figures A.1 and A.2). A subset of trinucleotide repeats, both anchored and unanchored, were screened (Figures A.3 and A.4).

Effect of Annealing Temperature on ISSRs

Many variables affect amplification in PCR. The concentration of reagents,

reaction volume, and temperature profile are three variables commonly optimized. When considering the temperature profile, modification of the annealing temperature causes the greatest change in amplification products. Primers bind to DNA during the annealing phase of PCR. In order to promote specific binding, i.e., where all bases match the template DNA, the annealing temperature (T_A) should be close to the melting temperature, at which the primer will dissociate from a complementary sequence. Lower temperatures may permit primer binding despite mismatches.

It is expected that bands amplified in low T_A reactions will disappear as the stringency is increased with increasing temperature, and at still higher temperatures, primers may no longer bind sufficiently to complementary sequences. This leads to a loss of bands at higher temperatures. Determining the optimum T_A involves balancing well-defined bands with loss of bands as T_A increases.

Comparing Reproducibility

ISSR is valued for its reproducibility when compared to RAPD, which uses a shorter primer, and low annealing temperatures. It is important to ensure loci are amplifiable and identical when compared across independent DNA extractions from the same source material.

Converting Gels to Numerical Data

One method for converting the gel data as seen in Figure 3.2 is based upon using the molecular ladder to generate a standard curve of distance migrated by a fragment of given length. Peaks of the lanes of interest are matched with this curve to calculate the size of fragments for that peak. Multiple lanes of ladder DNA can account for any

variation in migration rates. Loci are then associated with the length of the fragment producing that band. Comparison of loci between the parent and offspring plants can then be used to calculate genetic distance.

Materials and Methods

Plant Material

Plants were maintained in greenhouse conditions at the University of Nebraska at Omaha. DNA was extracted from *A. hewittii* leaf lamina tissue. Leaf midribs were removed before processing.

DNA Extraction

One effective protocol for plant DNA extraction is based on Cetyl Trimethyl Ammonium Bromide, commonly known as CTAB (Doyle and Doyle 1987). This procedure was scaled down proportionally for 0.1-gram samples. Upon isolation, genomic DNA was then checked for quality by measuring the absorbance ratio (A_{260}/A_{280}) of the sample. The concentration was normalized to 10ng/μL for PCR reactions.

PCR Conditions

PCR was carried out in 25 μL volumes with 2.5 μL 10x PCR buffer (Invitrogen), 0.75 μL 50 mM $MgCl_2$ (final concentration 1.5 mM), 10 ng template DNA, 0.5 U Taq Polymerase (Invitrogen), 1 μL 10 mM dNTP mixture (final concentration 1 mM each), and 100 pmol custom-made ISSR primer (IDT DNA). Reactions conditions were as follows: denaturation for 6 minutes at 94°C, cycled 32 times (1 minute at 94°C, 1 minute

at the designated annealing temperature, 2 minutes at 72°C), and followed by a final extension for 7 minutes at 72°C before electrophoresis.

Electrophoresis of PCR Products

Samples were mixed in a 10:1 ratio with Promega loading dye and 0.2 μL 100x SYBR Green dye (Bio-Rad). All gels were 1.2-1.5% agarose SB (sodium borate, 200 mM) as described by Brody and Kern (2004) and samples were electrophoresed at 250 volts. Molecular ladders used include λ phage digested with *Hind*III and *Eco*RI restriction enzymes and a 100-base ladder. Gel images were captured and processed as described in Appendix I.

Initial Primer Screening

Standard ISSR conditions were used to amplify loci for all primers (Figure 3.1). Annealing temperature was 45°C to promote annealing. Primers amplifying multiple, distinct bands when visually scored were selected for further characterization. See Tables 3.1 and 3.2 for a list of primers and bands amplified during screening. Lower discrimination by visual scoring probably undercounts the actual number of bands

Figure 3.1. Initial screening of ISSR primers. Sixteen primers are screened. Note that the unanchored primers have weak amplification as compared to other primers with the same repeat and an anchor nucleotide. Molecular ladders are λ phage DNA digested with *Hind*III and *Eco*RI.

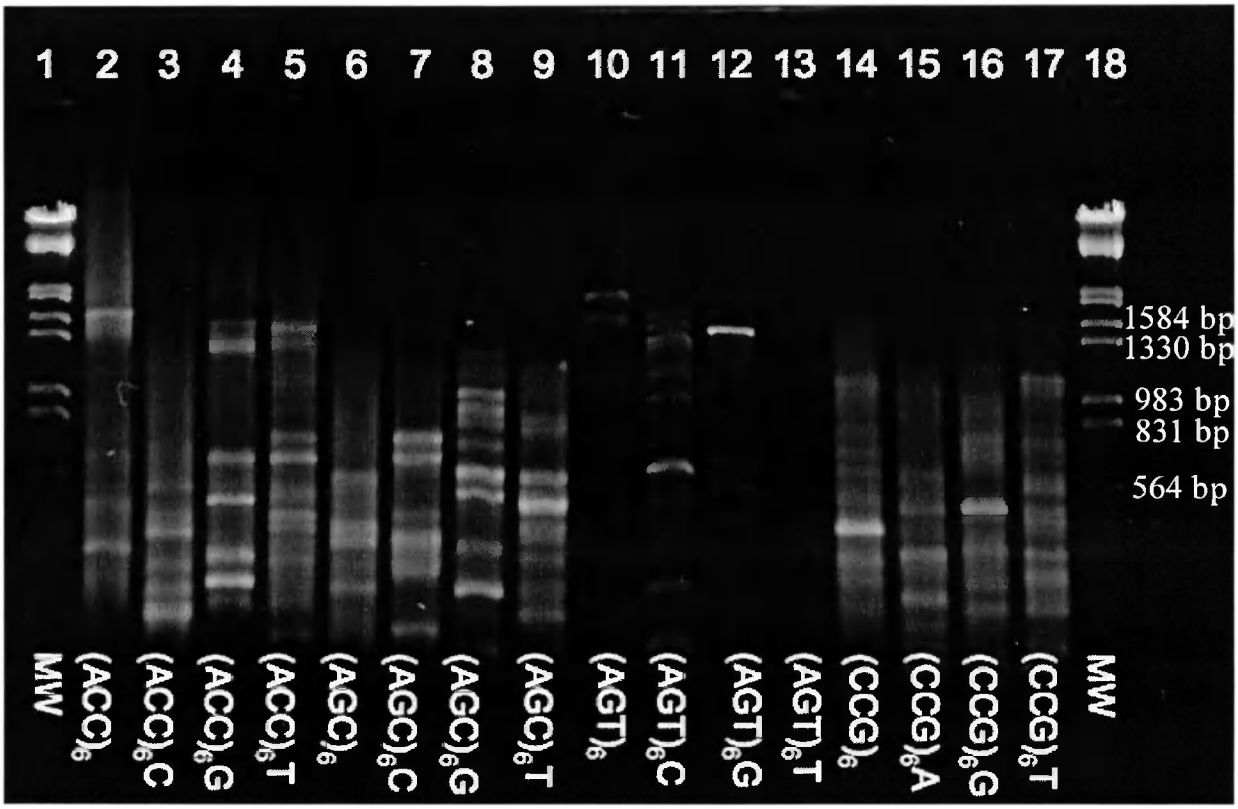


Table 3.1. Dinucleotide repeat ISSR primers screened. Also listed is the number of visually scorable loci for each primer. The actual number of amplified DNA fragments may be significantly higher. Total number of bands: 82. Average loci per primer: 3.0.

Primer	Loci	Primer	Loci	Primer	Loci
$(AC)_8$	0	$(AG)_8$	0	$(CT)_8$	0
$(AC)_8C$	2	$(AG)_8C$	3	$(CT)_8A$	5
$(AC)_8G$	6	$(AG)_8G$	2	$(CT)_8G$	7
$(AC)_8T$	5	$(AG)_8T$	5	$(CT)_8T$	5
$(CA)_8A$	4	$(GA)_8A$	4	$(TC)_8A$	5
$(CA)_8G$	5	$(GA)_8C$	5	$(TC)_8C$	6
$(CA)_8T$	6	$(GA)_8T$	3	$(TC)_8G$	4

Table 3.2. Trinucleotide repeat ISSR primers screened. Also listed is the number of visually scorable loci for each primer. The actual number of amplified DNA fragments may be significantly higher. Total number of bands: 206. Average loci per primer: 6.4.

Primer	Loci	Primer	Loci	Primer	Loci	Primer	Loci
$(ACC)_6$	4	$(AGT)_6$	2	$(CTC)_6$	4	$(GTT)_6$	3
$(ACC)_6C$	7	$(AGT)_6C$	12	$(CTC)_6A$	3	$(GTT)_6A$	9
$(ACC)_6G$	7	$(AGT)_6G$	6	$(CTC)_6G$	5	$(GTT)_6C$	7
$(ACC)_6T$	10	$(AGT)_6T$	0	$(CTC)_6T$	8	$(GTT)_6T$	7
$(AGC)_6$	5	$(CCG)_6$	9	$(GAA)_6$	7	$(TGC)_6$	0
$(AGC)_6C$	6	$(CCG)_6A$	9	$(GAA)_6A$	7	$(TGC)_6A$	0
$(AGC)_6G$	9	$(CCG)_6G$	9	$(GAA)_6C$	9	$(TGC)_6C$	7
$(AGC)_6T$	10	$(CCG)_6T$	11	$(GAA)_6T$	8	$(TGC)_6G$	6

Fifty-nine dinucleotide and trinucleotide repeat ISSR were screened; 288 loci were amplified with an average of 4.88 per primer. Unanchored primers typically amplified fewer bands than anchored primers. On average, trinucleotide repeat primers yielded more scorable bands than dinucleotide repeat primers (averages of 6.4 and 3.0, respectively).

Annealing Temperature Optimization

Selected primers from the initial screening were subjected to an annealing temperature (T_A) gradient to determine optimum PCR conditions (Figures A.5-A.16). Ideal T_A was selected by comparing the number and clarity of bands among lanes. A summary of annealing temperatures is shown in Table 3.3.

Table 3.3. Optimum annealing temperatures. Primers selected from the initial screen were run on a gradient of temperatures from 45°C to 55°C.

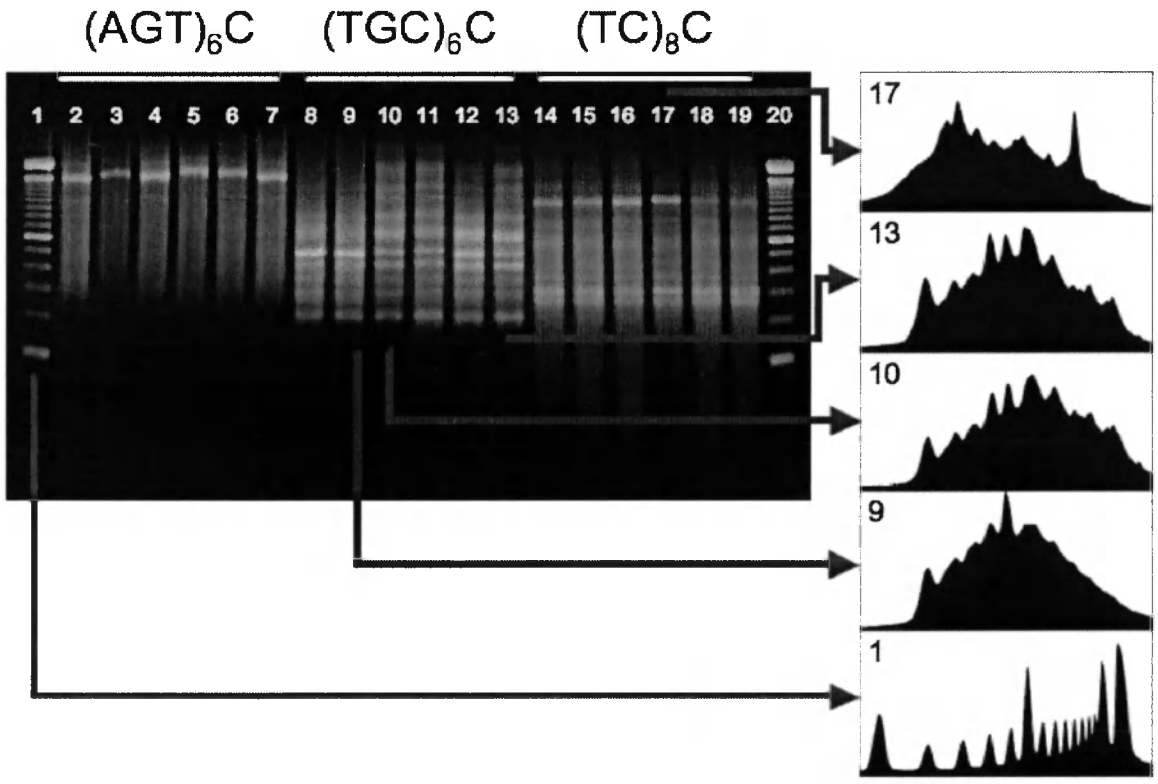
Primer	TA	Primer	TA	Primer	TA	Primer	TA
$(AG)_8A$	51°C	$(CA)_8A$	55°C	$(AGC)_6G$	49°C	$(GAA)_6C$	51°C
$(GA)_8A$	55°C	$(CA)_8G$	53°C	$(AGC)_6T$	55°C	$(GTT)_6A$	45°C
$(GA)_8C$	49°C	$(CA)_8T$	51°C	$(AGT)_6C$	49°C	$(GTT)_6C$	47°C
$(CT)_8A$	51°C	$(AC)_8G$	55°C	$(AGT)_6G$	47°C	$(GTT)_6T$	45°C
$(CT)_8G$	49°C	$(AC)_8T$	55°C	$(CCG)_6A$	47°C	$(TGC)_6C$	45°C
$(CT)_8T$	49°C	$(ACC)_6C$	51°C	$(CCG)_6G$	53°C	$(TGC)_6G$	55°C
$(TC)_8A$	49°C	$(ACC)_6G$	51°C	$(CCG)_6T$	47°C		
$(TC)_8C$	47°C	$(ACC)_8T$	49°C	$(CTC)_6G$	45°C		
$(TC)_8G$	47°C	$(AGC)_8C$	51°C	$(GAA)_6A$	55°C		

Parent / Clone Comparison

Products of PCR reactions containing genomic DNA of either the parent plant or one of the offspring were next separated by gel electrophoresis. A 100 base-pair ladder was used as a baseline for estimating fragment size. Two replicates of independent DNA

extractions demonstrate reproducibility (Figure A.17). Lanes were converted to linear plots using ImageJ and banding patterns compared (Figures 3.2 and A.18). This method is far more sensitive and accurate than visually scoring the gel. Any increase in signal that showed clear demarcation and exceeded three percent on both sides was scored as a locus.

Figure 3.2. Gel conversion to an intensity graph. Lanes are grouped by primer. Paired lanes with each primer group include two independent DNA extractions for the parent plant (first two lanes) and duplicates of two clones from the *in vitro* protocol as the second and third pairs, respectively. Lanes 1 and 20 contain a 100 base-pair ladder with 100, 600, and 1500 bases markers of higher intensity.



Results and Discussion

The ISSR method was able to amplify sufficient loci for a distance analysis of *Amorphophallus hewittii*. Proof-of-concept screening of three primers did not uncover any genetic mutations of the amplified areas, but a larger population of offspring and more loci are required for a meaningful analysis of mutation rates. Research on banana plants, (*Musa acuminata* var. Nanjanagudi Rasabale) in culture for approximately 10 years, generated 424 bands with RAPD and ISSR. They examined the mother plant and eleven offspring and found no differences in loci as compared to plants not subjected to tissue culture (Lakshmanan et al. 2007).

In population studies, both monomorphic (demonstrated in all individuals) and polymorphic (those that vary between individuals) markers are useful. Polymorphism could not be determined during the initial primer screen as there was only one individual screened. But with the hypervariable nature of ISSRs and the sheer number of loci amplified during the primer screen, sufficient loci (both polymorphic and monomorphic) for population and systematic studies could be amplified with the existing primers.

The primer screen of *Amorphophallus hewittii* uncovered a general trend for primers based on a $(CT)_8N$ or one of the tri-nucleotide repeats such as $(AGC)_6N$, $(ACC)_6N$, and $(CTC)_6N$ to amplify multiple clear bands with good intensity. Unanchored primers within a repeat family tended to yield fewer strongly-amplified loci than anchored primers. This may be an indication that the primers are not selective enough. Further restrictions on the annealing sequence such as using a dinucleotide anchor at the 3` end would allow for further segregation of these bands between primers and uncover

sites that are less strongly amplified.

Background noise is present at relatively high levels. Better resolution and improved amplification may be possible through a second round of PCR using a dilution of the first round PCR product as the template DNA.

Conclusions

With the primers screened, sufficient loci exist for most applications where randomly amplified primers are suitable. An investigation focusing on resolving additional loci with primers terminating in more anchoring nucleotides would determine if the background noise is an artifact of too many loci.

This work represents the first set of ISSR markers optimized for *Amorphophallus hewittii*. Their utility may be extended for studies of naturally-occurring genetic diversity and it is conceivable that they may be used in breeding programs for agriculturally significant *Amorphophallus* species. These markers will also permit the exploration of taxonomy in the genus if the optimized markers are employed on other species. The amplified DNA fragments may also provide a stepping-stone to develop SSR markers to explore heterozygosity and homozygosity in the species.

Appendix I: Gel Images

Notes on the Images

All of the images in this appendix were taken with a Canon digital camera. The SYBR green dye was excited using a UV box. All images were photographed through a yellow photographic filter with an exposure of one to three seconds. Camera images in JPEG format were then processed with ImageJ 1.37 (available from the National Institute of Health). The workflow is as follows:

Rotate image to align gel with screen if necessary.

Crop image at gel edges.

Split the red, green, and blue (RGB) layers.

Discard the red and blue layers.

Execute the Gel Converter (v 1.0) plugin available from

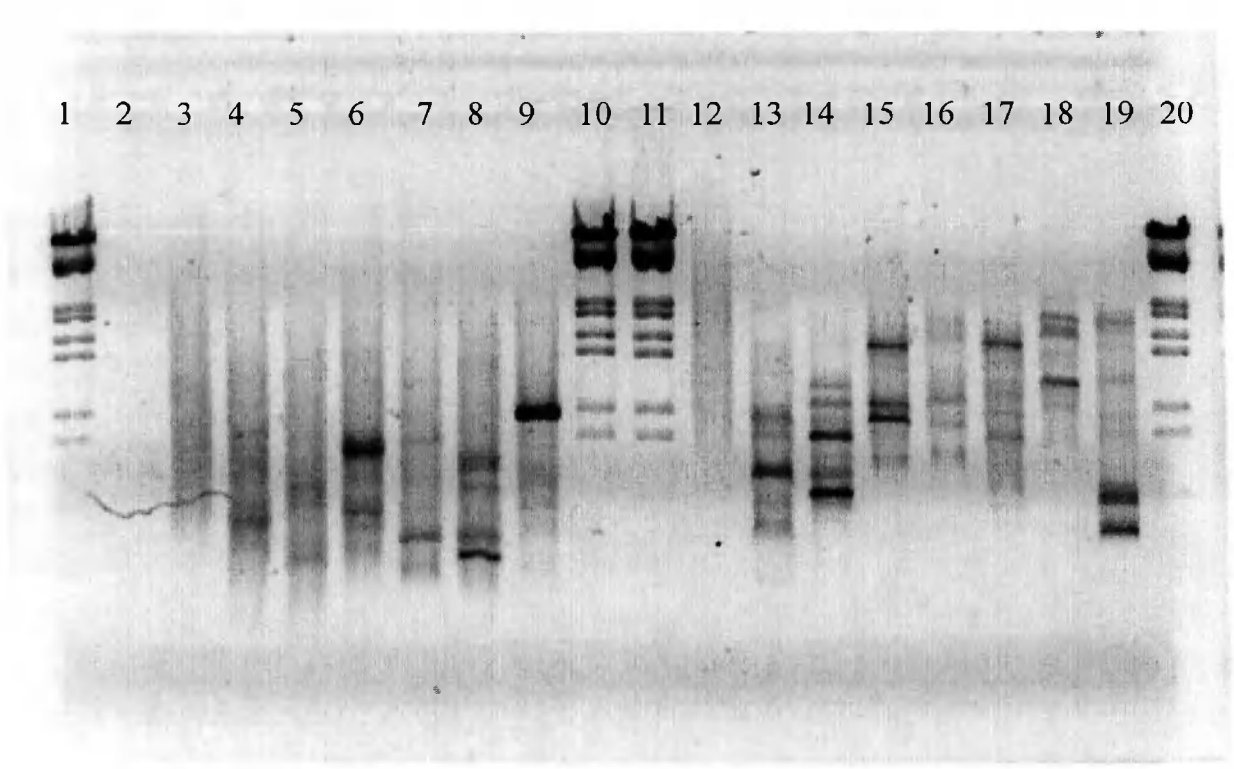
http://www.virginia.edu/biology/Fac/hirsh_gel/gel/Gel_converter.class

Invert the LUT (look up table) to set the background to white.

Save image as an uncompressed TIF file.

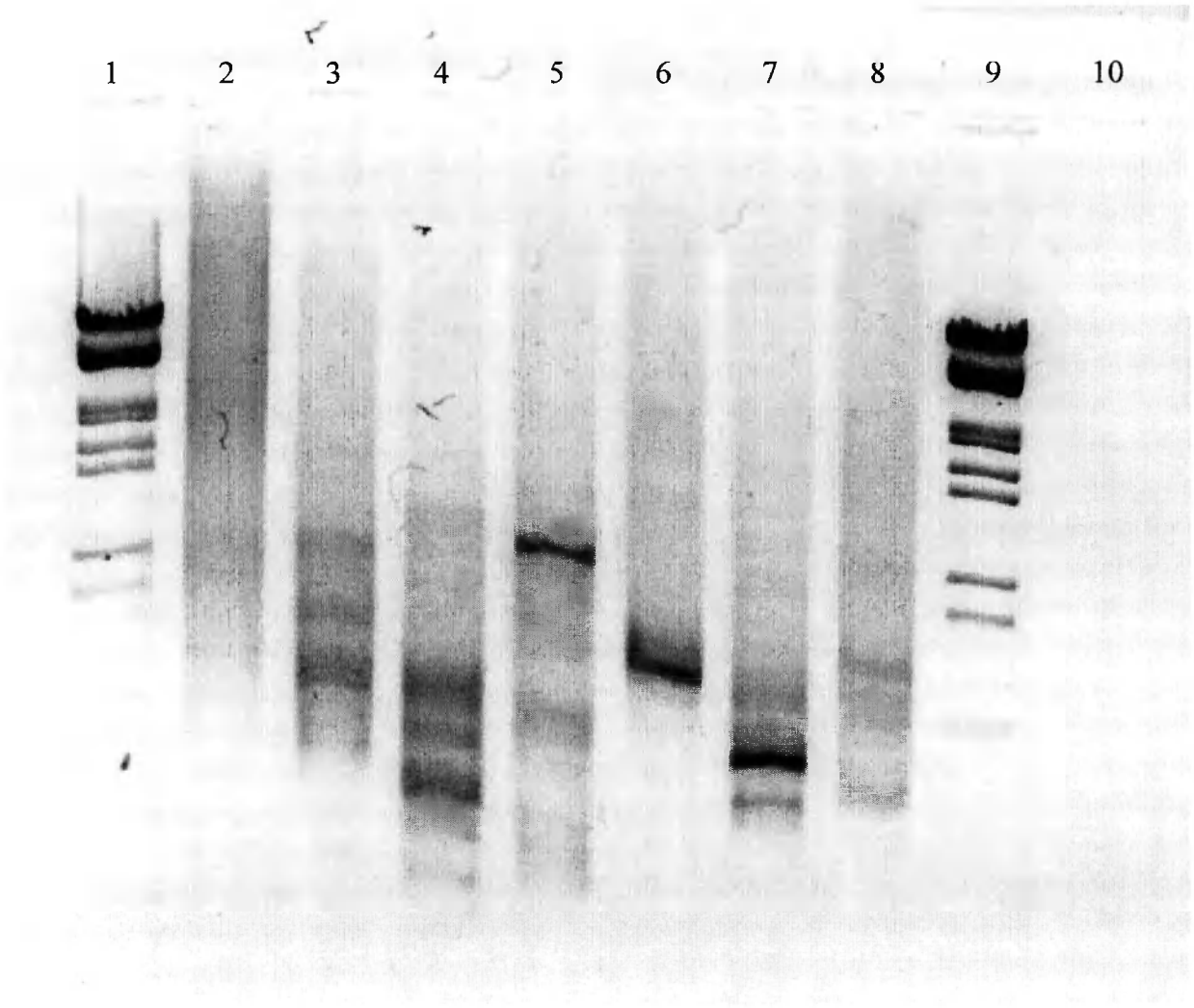
Figure A.18 was generated using the Gel Analysis package of ImageJ. Only a medial portion of the band was highlighted for processing to avoid distortion from electrophoresis. Area under the curves was filled using the bucket fill tool. Images were then saved to JPEG format.

Figure A.1. Primer Screen #1.



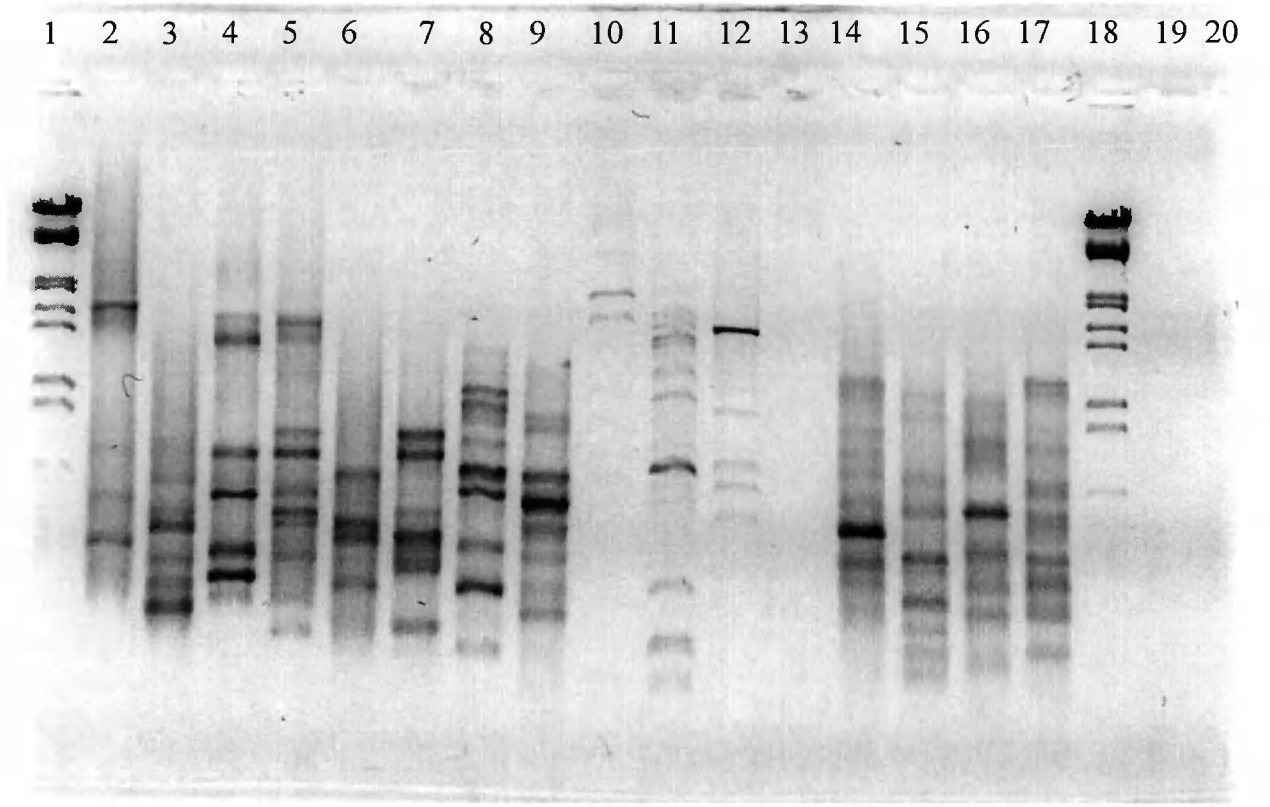
Lane	Contents	Lane	Contents
1	Ladder	11	Ladder
2	Neg. Control	12	$(CT)_8$
3	$(AG)_8$	13	$(CT)_8A$
4	$(AG)_8C$	14	$(CT)_8G$
5	$(AG)_8G$	15	$(CT)_8T$
6	$(AG)_8T$	16	$(TC)_8A$
7	$(GA)_8A$	17	$(TC)_8C$
8	$(GA)_8C$	18	$(TC)_8G$
9	$(GA)_8T$	19	$(CTC)_5$
10	Ladder	20	Ladder

Figure A.2. Primer Screen #2.



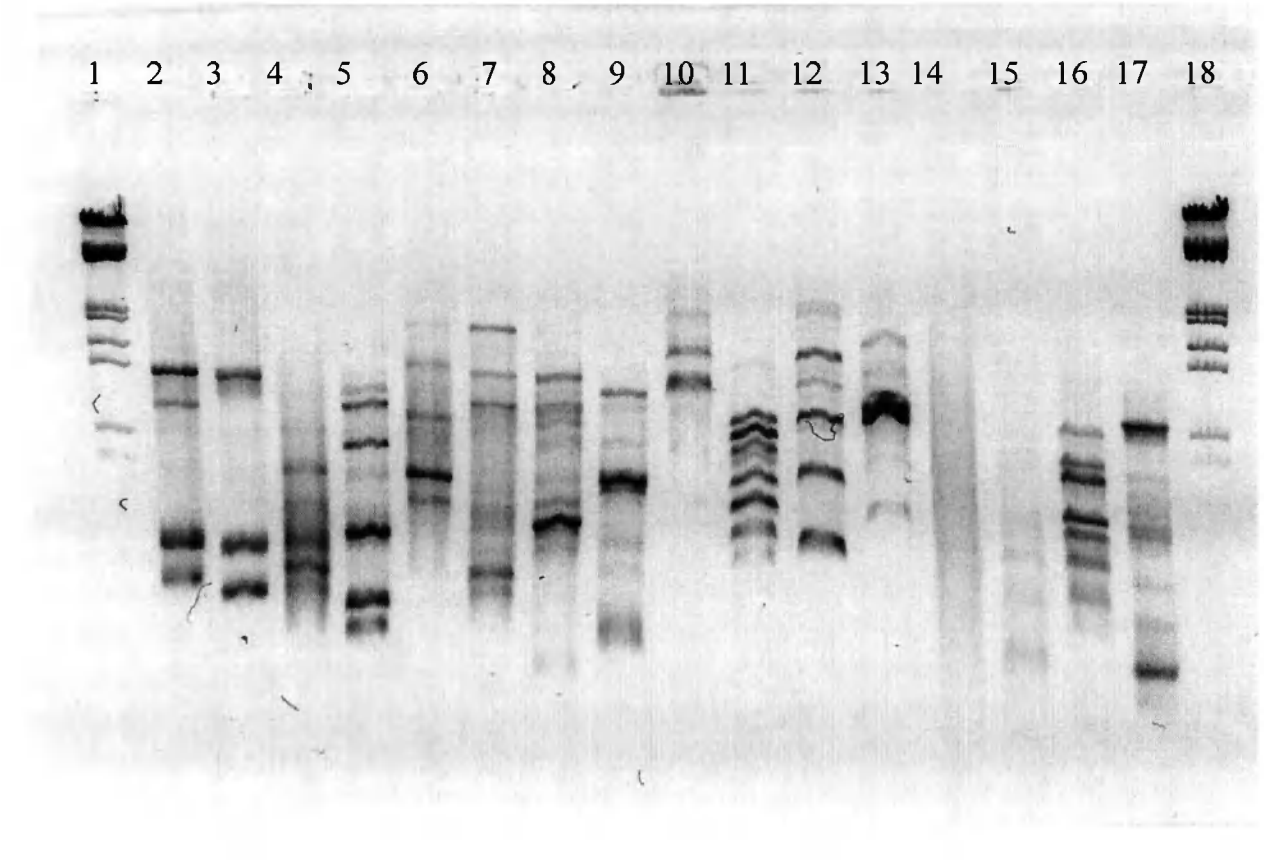
Lane	Contents	Lane	Contents
1	Ladder	6	$(AC)_8C$
2	$(CA)_8$	7	$(AC)_8G$
3	$(CA)_8A$	8	$(AC)_8T$
4	$(CA)_8G$	9	Ladder
5	$(CA)_8T$	10	--

Figure A.3. Primer Screen #3.



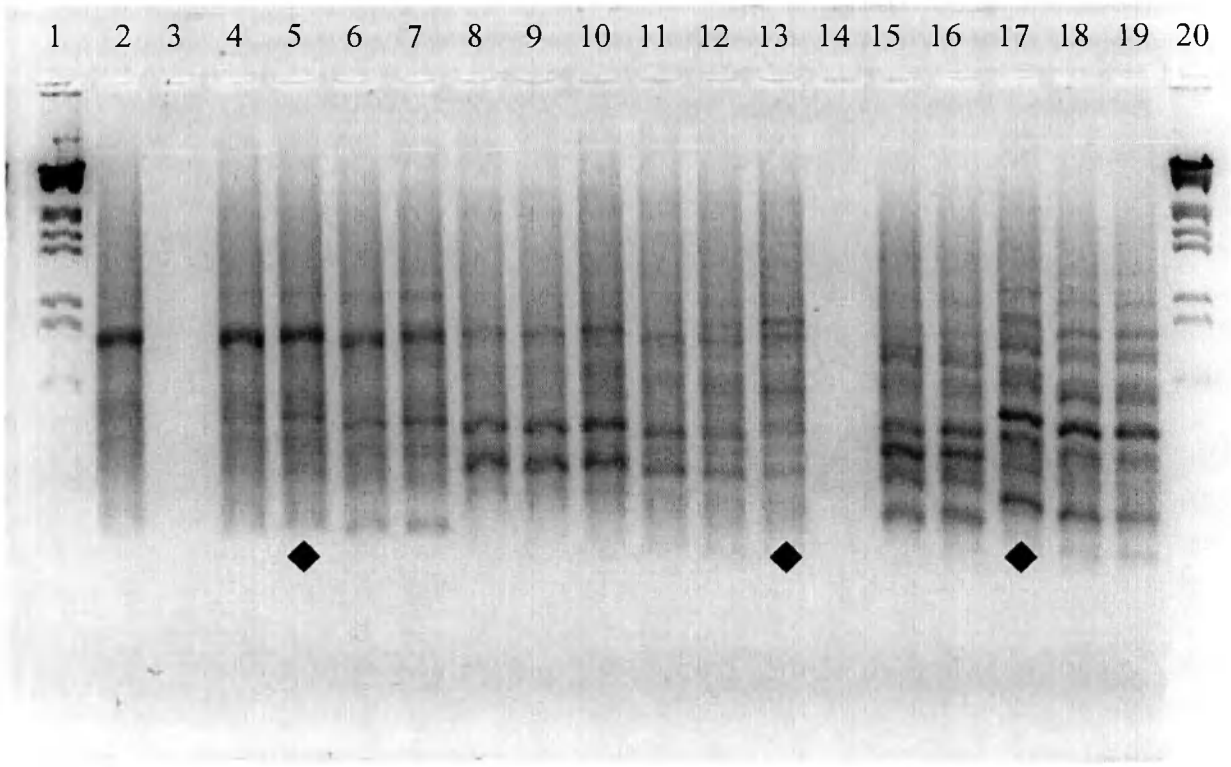
Lane	Contents	Lane	Contents
1	Ladder	11	$(AGT)_6C$
2	$(ACC)_6$	12	$(AGT)_6G$
3	(ACC)6C	13	$(AGT)_6T$
4	$(ACC)_6G$	14	$(CCG)_6$
5	$(ACC)_6T$	15	$(CCG)_6A$
6	$(AGC)_6$	16	$(CCG)_6G$
7	$(AGC)_6C$	17	$(CCG)_6T$
8	$(AGC)_6G$	18	Ladder
9	$(AGC)_6T$	19	--
10	$(AGT)_6$	20	--

Figure A.4. Primer Screen #4.



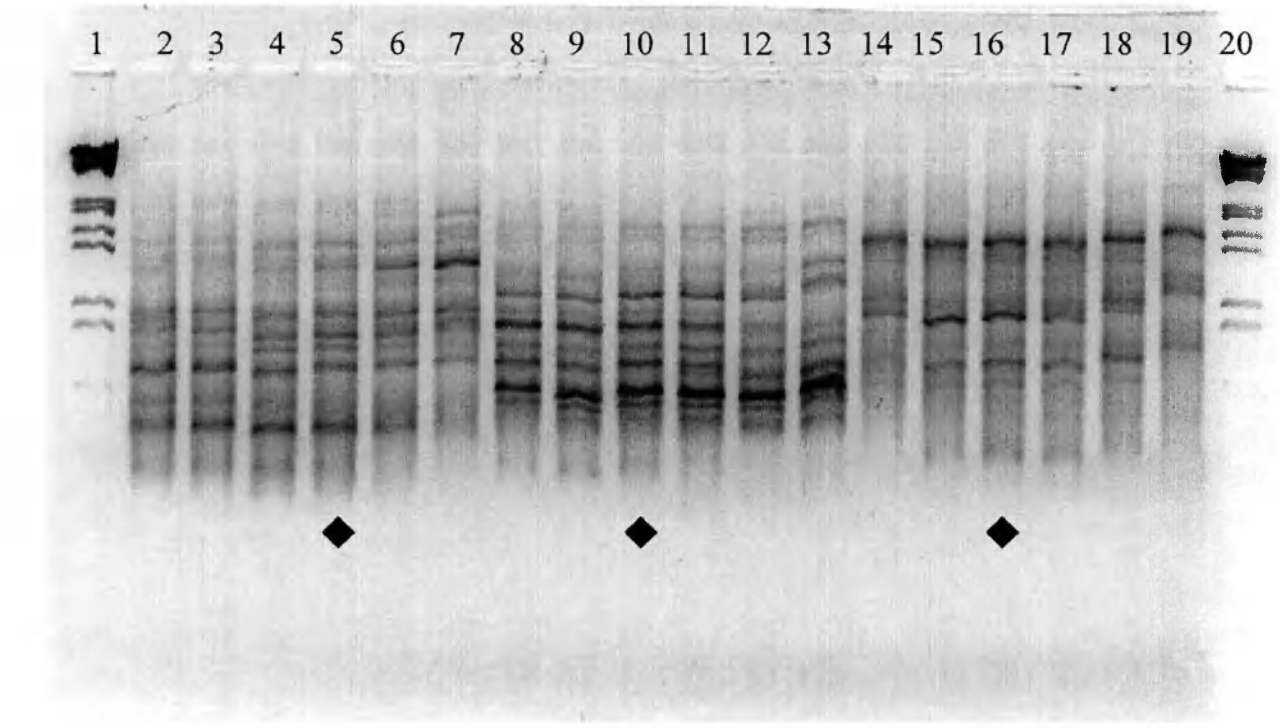
Lane	Contents	Lane	Contents
1	Ladder	10	$(GTT)_6$
2	$(CTC)_6$	11	$(GTT)_6A$
3	$(CTC)_6A$	12	$(GTT)_6C$
4	$(CTC)_6G$	13	$(GTT)_6T$
5	$(CTC)_6T$	14	$(TGC)_6$
6	$(GAA)_6$	15	$(TGC)_6A$
7	$(GAA)_6A$	16	$(TGC)_6C$
8	$(GAA)_6C$	17	$(TGC)_6G$
9	$(GAA)_6T$	18	Ladder

Figure A.5. Annealing Temperature (T_a) Screen #1. Diamonds denote optimum T_A.



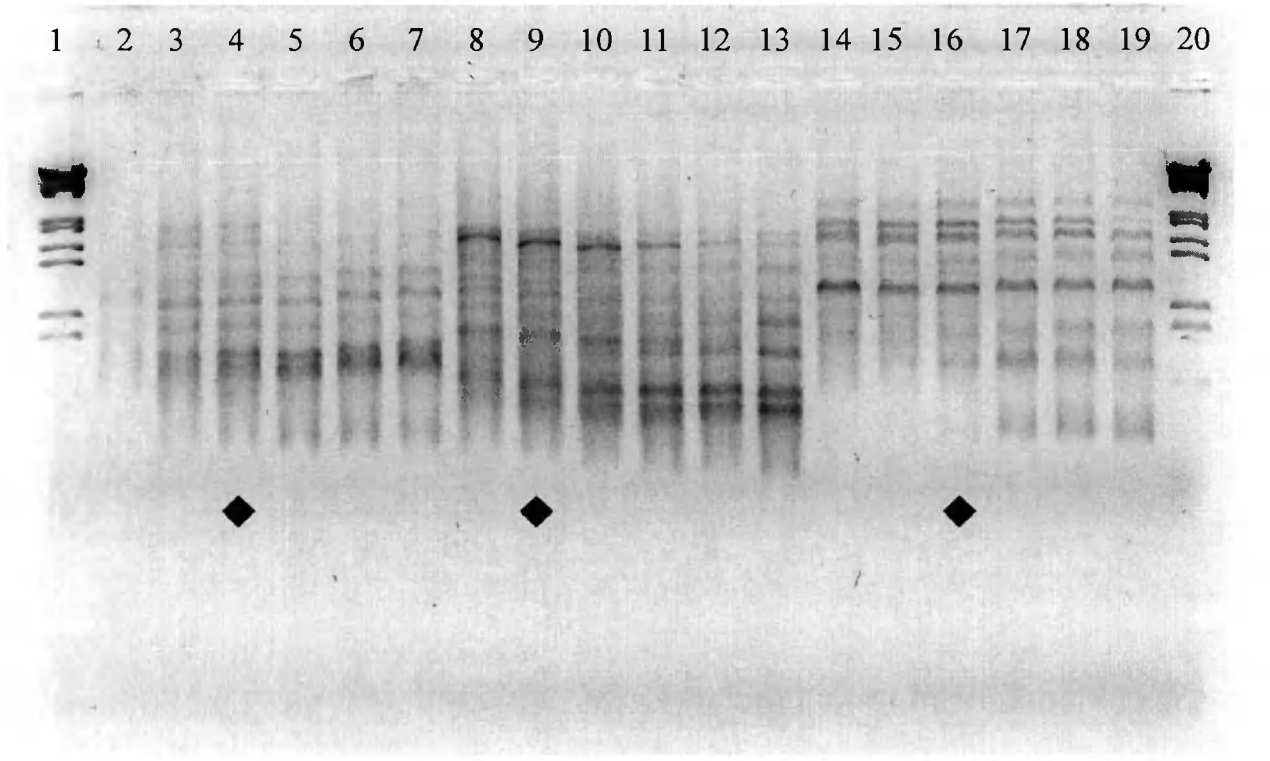
Lane	Primer / T_a	Lane	Primer / T_a
1	Ladder	11	$(GA)_8A$ / 51°C
2	$(AG)_8T$ / 45°C	12	$(GA)_8A$ / 53°C
3	$(AG)_8T$ / 47°C	13	$(GA)_8A$ / 55°C
4	$(AG)_8T$ / 49°C	14	$(GA)_8C$ / 45°C
5	$(AG)_8T$ / 51°C	15	$(GA)_8C$ / 47°C
6	$(AG)_8T$ / 53°C	16	$(GA)_8C$ / 49°C
7	$(AG)_8T$ / 55°C	17	$(GA)_8C$ / 51°C
8	$(GA)_8A$ / 45°C	18	$(GA)_8C$ / 53°C
9	$(GA)_8A$ / 47°C	19	$(GA)_8C$ / 55°C
10	$(GA)_8A$ / 49°C	20	Ladder

Figure A.6. Annealing Temperature (T_a) Screen #2. Diamonds denote optimum T_A.



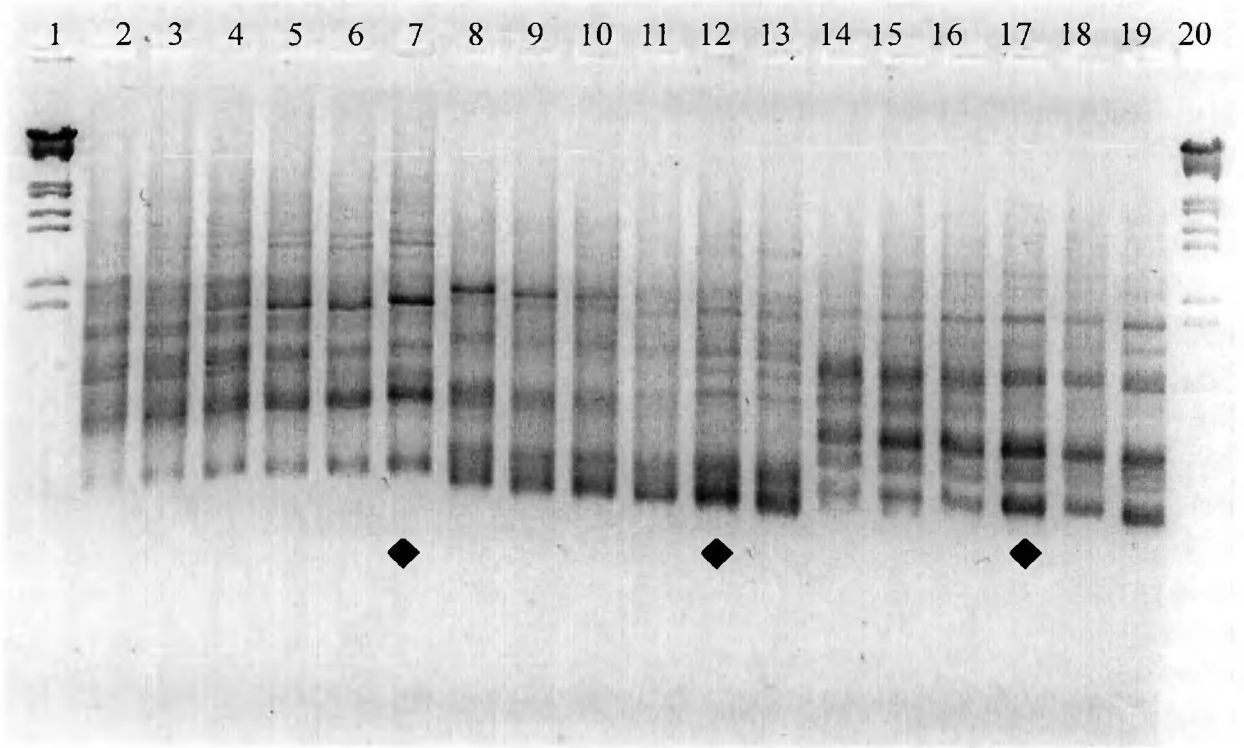
Lane	Primer / T_a	Lane	Primer / T_a
1	Ladder	11	$(CT)_8G$ / 51°C
2	$(CT)_8A$ / 45°C	12	$(CT)_8G$ / 53°C
3	$(CT)_8A$ / 47°C	13	$(CT)_8G$ / 55°C
4	$(CT)_8A$ / 49°C	14	$(CT)_8T$ / 45°C
5	$(CT)_8A$ / 51°C	15	$(CT)_8T$ / 47°C
6	$(CT)_8A$ / 53°C	16	$(CT)_8T$ / 49°C
7	$(CT)_8A$ / 55°C	17	$(CT)_8T$ / 51°C
8	$(CT)_8G$ / 45°C	18	$(CT)_8T$ / 53°C
9	$(CT)_8G$ / 47°C	19	$(CT)_8T$ / 55°C
10	$(CT)_8G$ / 49°C	20	Ladder

Figure A.7. Annealing Temperature (T_a) Screen #3. Diamonds denote optimum T_A.



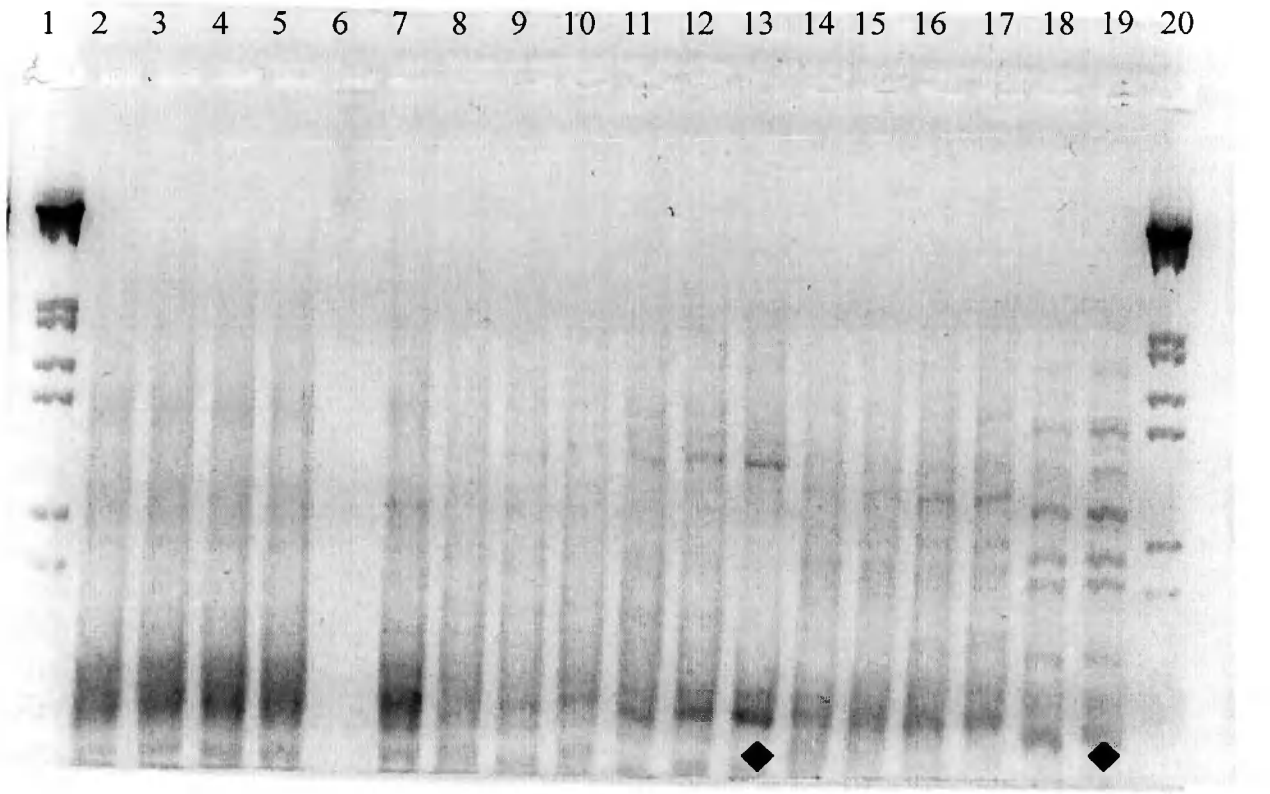
Lane	Primer / T_a	Lane	Primer / T_a
1	Ladder	11	$(TC)_8C$ / 51°C
2	$(TC)_8A$ / 45°C	12	$(TC)_8C$ / 53°C
3	$(TC)_8A$ / 47°C	13	$(TC)_8C$ / 55°C
4	$(TC)_8A$ / 49°C	14	$(TC)_8G$ / 45°C
5	$(TC)_8A$ / 51°C	15	$(TC)_8G$ / 47°C
6	$(TC)_8A$ / 53°C	16	$(TC)_8G$ / 49°C
7	$(TC)_8A$ / 55°C	17	$(TC)_8G$ / 51°C
8	$(TC)_8C$ / 45°C	18	$(TC)_8G$ / 53°C
9	$(TC)_8C$ / 47°C	19	$(TC)_8G$ / 55°C
10	$(TC)_8C$ / 49°C	20	Ladder

Figure A.8. Annealing Temperature (T_a) Screen #4. Diamonds denote optimum T_A.



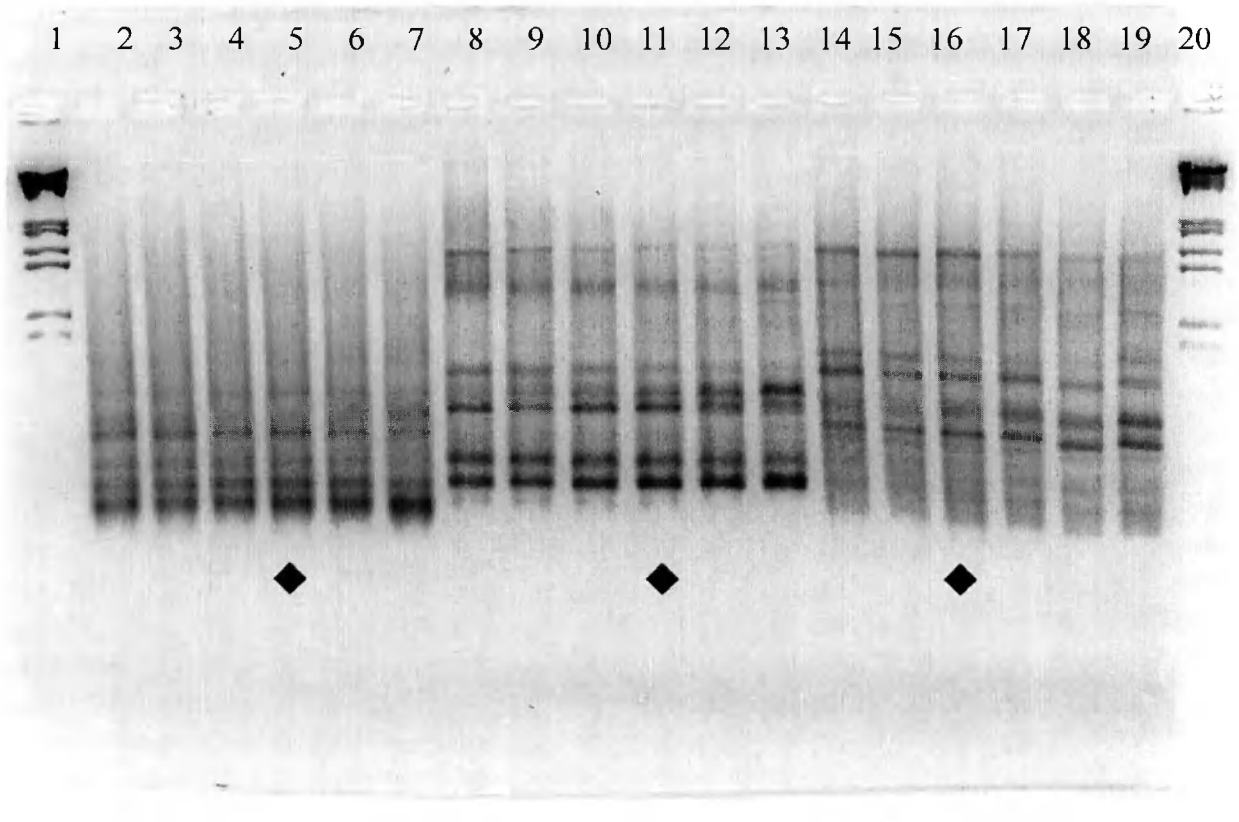
Lane	Primer / T_a	Lane	Primer / T_a
1	Ladder	11	$(CA)_8G$ / 51°C
2	$(CA)_8A$ / 45°C	12	$(CA)_8G$ / 53°C
3	$(CA)_8A$ / 47°C	13	$(CA)_8G$ / 55°C
4	$(CA)_8A$ / 49°C	14	$(CA)_8T$ / 45°C
5	$(CA)_8A$ / 51°C	15	$(CA)_8T$ / 47°C
6	$(CA)_8A$ / 53°C	16	$(CA)_8T$ / 49°C
7	$(CA)_8A$ / 55°C	17	$(CA)_8T$ / 51°C
8	$(CA)_8G$ / 45°C	18	$(CA)_8T$ / 53°C
9	$(CA)_8G$ / 47°C	19	$(CA)_8T$ / 55°C
10	$(CA)_8G$ / 49°C	20	Ladder

Figure A.9. Annealing Temperature (T_a) Screen #5. Diamonds denote optimum T_A.



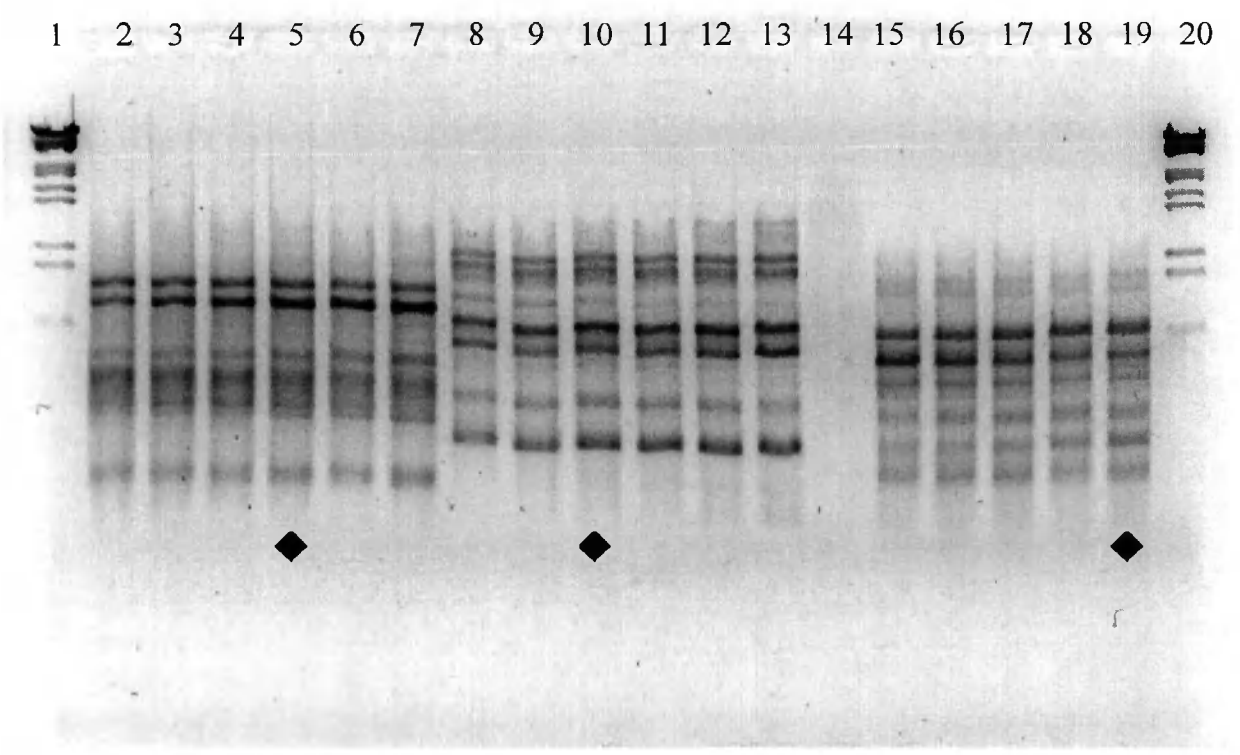
Lane	Primer / T_a	Lane	Primer / T_a
1	Ladder	11	$(AC)_8G$ / 51°C
2	$(AC)_8C$ / 45°C	12	$(AC)_8G$ / 53°C
3	$(AC)_8C$ / 47°C	13	$(AC)_8G$ / 55°C
4	$(AC)_8C$ / 49°C	14	$(AC)_8T$ / 45°C
5	$(AC)_8C$ / 51°C	15	$(AC)_8T$ / 47°C
6	$(AC)_8C$ / 53°C	16	$(AC)_8T$ / 49°C
7	$(AC)_8C$ / 55°C	17	$(AC)_8T$ / 51°C
8	$(AC)_8G$ / 45°C	18	$(AC)_8T$ / 53°C
9	$(AC)_8G$ / 47°C	19	$(AC)_8T$ / 55°C
10	$(AC)_8G$ / 49°C	20	Ladder

Figure A.10. Annealing Temperature (T_a) Screen #6. Diamonds denote optimum T_A.



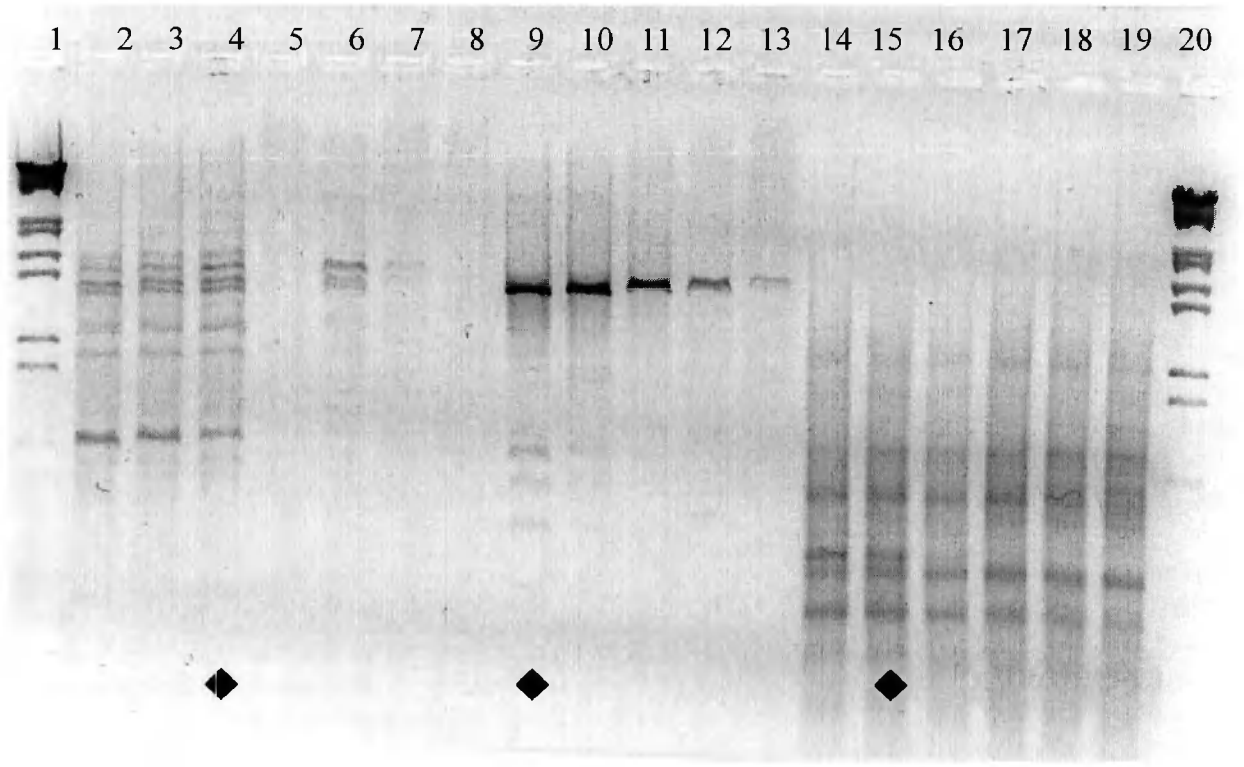
Lane	Primer / T_a	Lane	Primer / T_a
1	Ladder	11	$(ACC)_6G$ / 51°C
2	$(ACC)_6C$ / 45°C	12	$(ACC)_6G$ / 53°C
3	$(ACC)_6C$ / 47°C	13	$(ACC)_6G$ / 55°C
4	$(ACC)_6C$ / 49°C	14	$(ACC)_6T$ / 45°C
5	$(ACC)_6C$ / 51°C	15	$(ACC)_6T$ / 47°C
6	$(ACC)_6C$ / 53°C	16	$(ACC)_6T$ / 49°C
7	$(ACC)_6C$ / 55°C	17	$(ACC)_6T$ / 51°C
8	$(ACC)_6G$ / 45°C	18	$(ACC)_6T$ / 53°C
9	$(ACC)_6G$ / 47°C	19	$(ACC)_6T$ / 55°C
10	$(ACC)_6G$ / 49°C	20	Ladder

Figure A.11. Annealing Temperature (T_a) Screen #7. Diamonds denote optimum T_A.



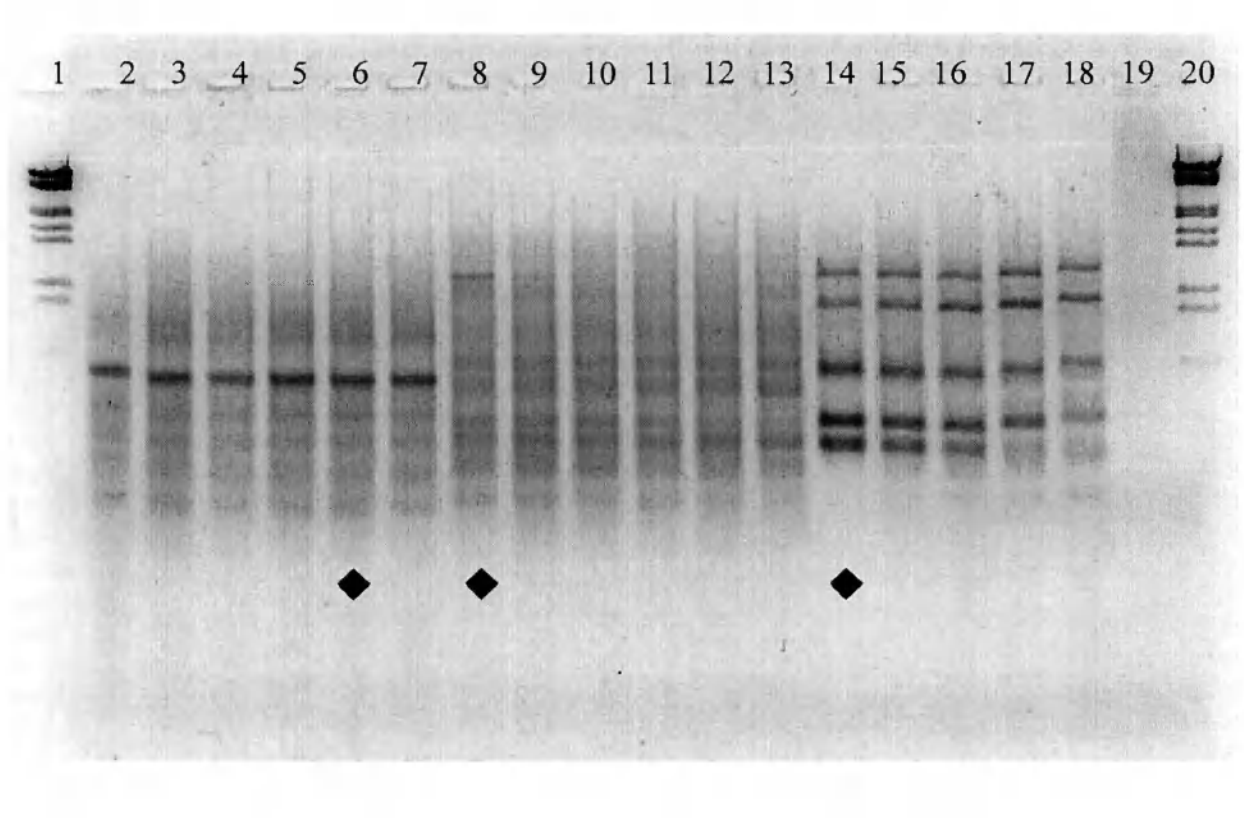
Lane	Primer / T_a	Lane	Primer / T_a
1	Ladder	11	$(AGC)_6G$ / 51°C
2	$(AGC)_6C$ / 45°C	12	$(AGC)_6G$ / 53°C
3	$(AGC)_6C$ / 47°C	13	$(AGC)_6G$ / 55°C
4	$(AGC)_6C$ / 49°C	14	$(AGC)_6T$ / 45°C
5	$(AGC)_6C$ / 51°C	15	$(AGC)_6T$ / 47°C
6	$(AGC)_6C$ / 53°C	16	$(AGC)_6T$ / 49°C
7	$(AGC)_6C$ / 55°C	17	$(AGC)_6T$ / 51°C
8	$(AGC)_6G$ / 45°C	18	$(AGC)_6T$ / 53°C
9	$(AGC)_6G$ / 47°C	19	$(AGC)_6T$ / 55°C
10	$(AGC)_6G$ / 49°C	20	Ladder

Figure A.12. Annealing Temperature (T_a) Screen #8. Diamonds denote optimum T_A.



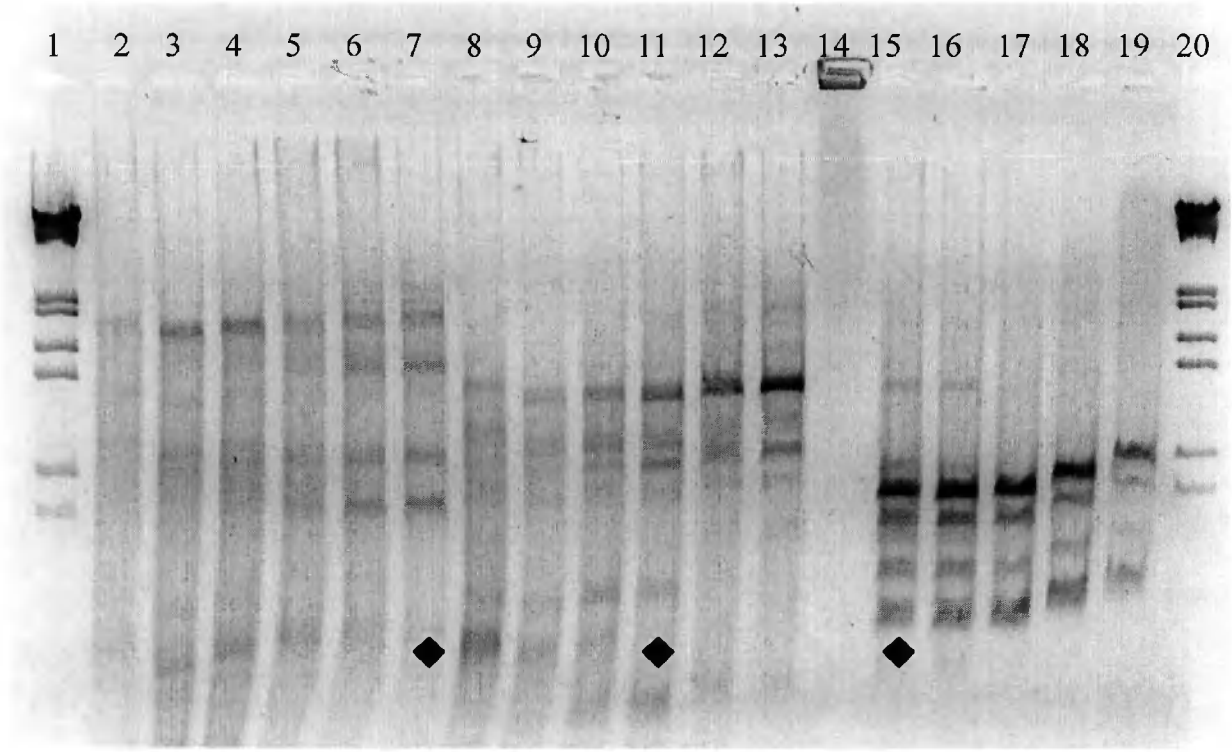
Lane	Primer / T_a	Lane	Primer / T_a
1	Ladder	11	$(AGT)_6G$ / 51°C
2	$(AGT)_6C$ / 45°C	12	$(AGT)_6G$ / 53°C
3	$(AGT)_6C$ / 47°C	13	$(AGT)_6G$ / 55°C
4	$(AGT)_6C$ / 49°C	14	$(CCG)_6A$ / 45°C
5	$(AGT)_6C$ / 51°C	15	$(CCG)_6A$ / 47°C
6	$(AGT)_6C$ / 53°C	16	$(CCG)_6A$ / 49°C
7	$(AGT)_6C$ / 55°C	17	$(CCG)_6A$ / 51°C
8	$(AGT)_6G$ / 45°C	18	$(CCG)_6A$ / 53°C
9	$(AGT)_6G$ / 47°C	19	$(CCG)_6A$ / 55°C
10	$(AGT)_6G$ / 49°C	20	Ladder

Figure A.13. Annealing Temperature (T_a) Screen #9. Diamonds denote optimum T_A.



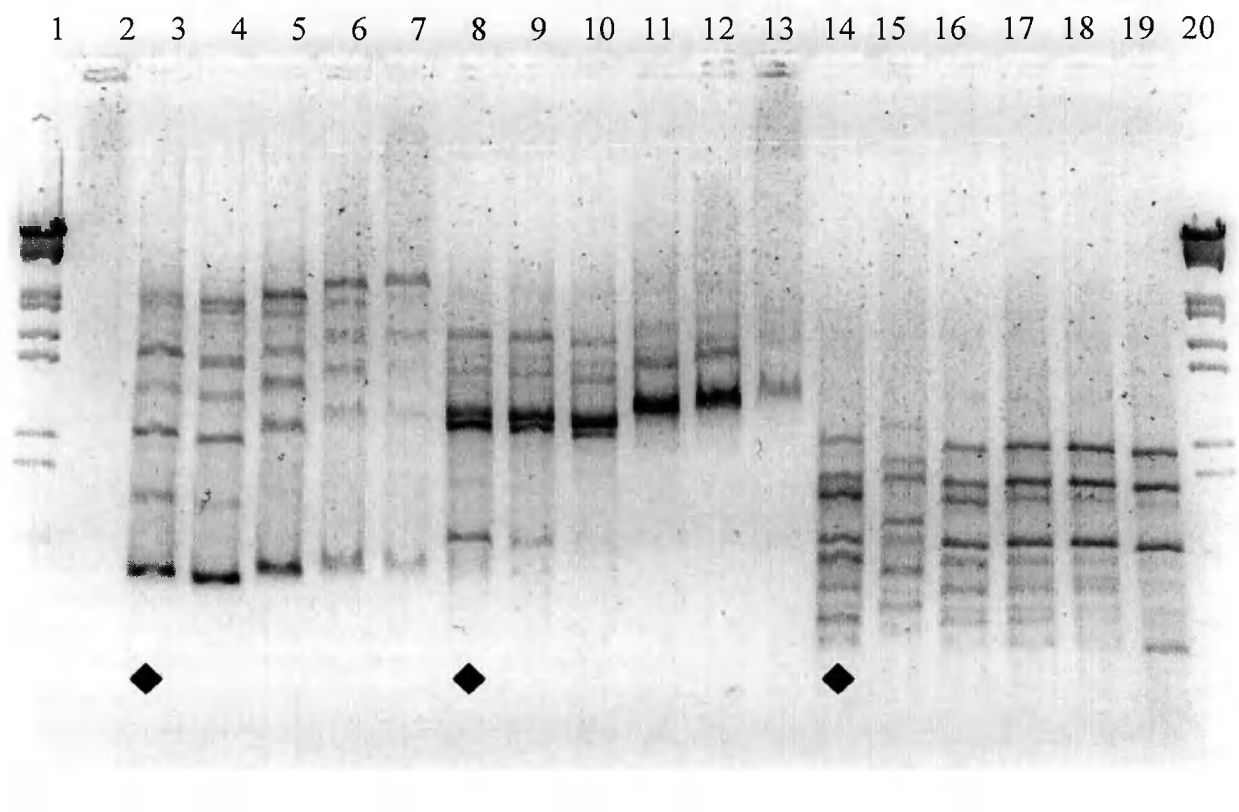
Lane	Primer / T_a	Lane	Primer / T_a
1	Ladder	11	$(CCG)_6T$ / 51°C
2	$(CCG)_6G$ / 45°C	12	$(CCG)_6T$ / 53°C
3	$(CCG)_6G$ / 47°C	13	$(CCG)_6T$ / 55°C
4	$(CCG)_6G$ / 49°C	14	$(CTC)_6G$ / 45°C
5	$(CCG)_6G$ / 51°C	15	$(CTC)_6G$ / 47°C
6	$(CCG)_6G$ / 53°C	16	$(CTC)_6G$ / 49°C
7	$(CCG)_6G$ / 55°C	17	$(CTC)_6G$ / 51°C
8	$(CCG)_6T$ / 45°C	18	$(CTC)_6G$ / 53°C
9	$(CCG)_6T$ / 47°C	19	$(CTC)_6G$ / 55°C
10	$(CCG)_6T$ / 49°C	20	Ladder

Figure A.14. Annealing Temperature (T_a) Screen #10. Diamonds denote optimum T_A.



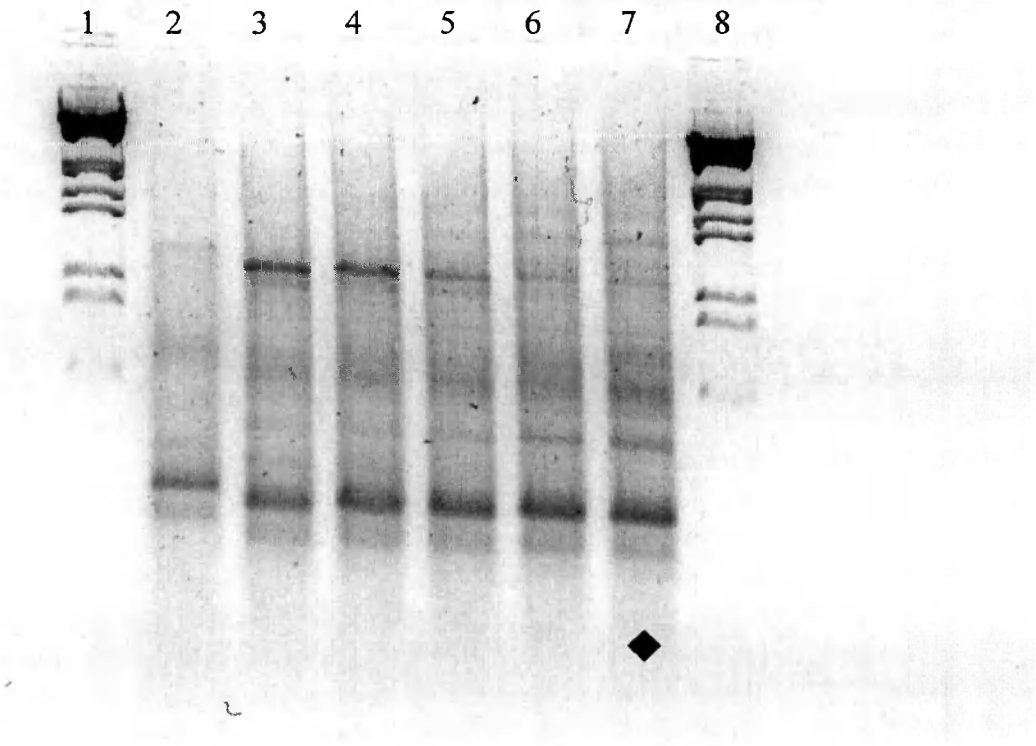
Lane	Primer / T_a	Lane	Primer / T_a
1	Ladder	11	$(GAA)_6C$ / 51°C
2	$(GAA)_6A$ / 45°C	12	$(GAA)_6C$ / 53°C
3	$(GAA)_6A$ / 47°C	13	$(GAA)_6C$ / 55°C
4	$(GAA)_6A$ / 49°C	14	$(GTT)_6A$ / 45°C
5	$(GAA)_6A$ / 51°C	15	$(GTT)_6A$ / 47°C
6	$(GAA)_6A$ / 53°C	16	$(GTT)_6A$ / 49°C
7	$(GAA)_6A$ / 55°C	17	$(GTT)_6A$ / 51°C
8	$(GAA)_6C$ / 45°C	18	$(GTT)_6A$ / 53°C
9	$(GAA)_6C$ / 47°C	19	$(GTT)_6A$ / 55°C
10	$(GAA)_6C$ / 49°C	20	Ladder

Figure A.15. Annealing Temperature (T_a) Screen #11. Diamonds denote optimum T_A.



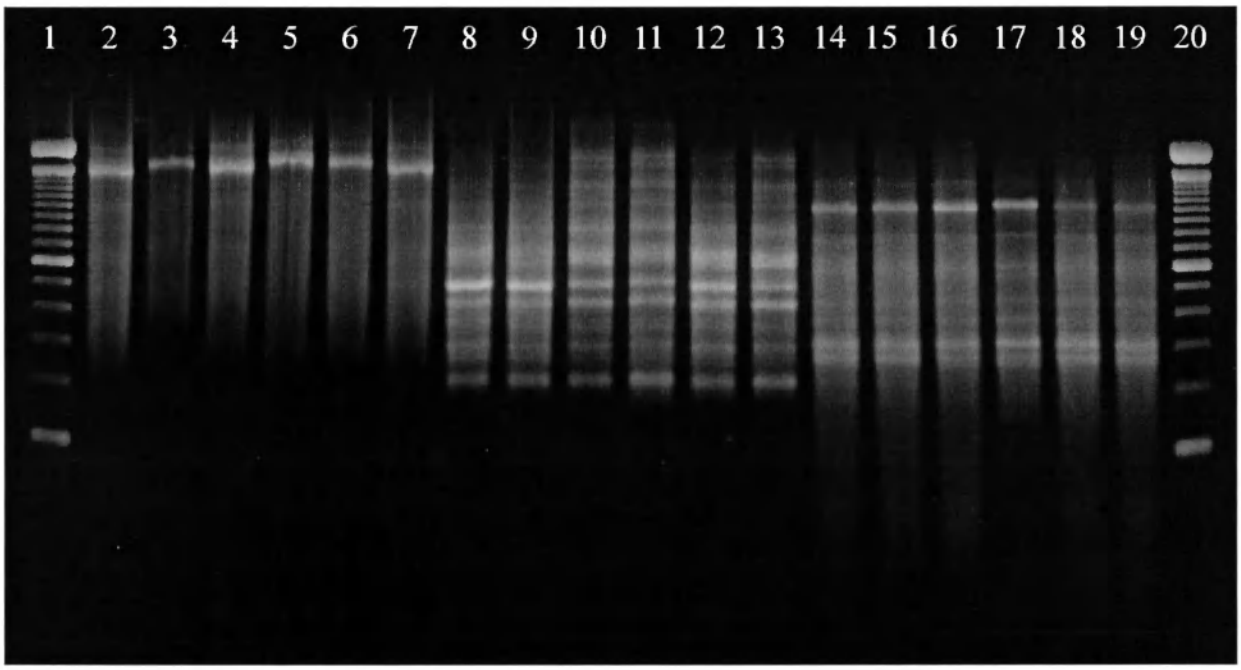
Lane	Primer / T_a	Lane	Primer / T_a
1	Ladder	11	$(GTT)_6T$ / 51°C
2	$(GTT)_6C$ / 45°C	12	$(GTT)_6T$ / 53°C
3	$(GTT)_6C$ / 47°C	13	$(GTT)_6T$ / 55°C
4	$(GTT)_6C$ / 49°C	14	$(TGC)_6C$ / 45°C
5	$(GTT)_6C$ / 51°C	15	$(TGC)_6C$ / 47°C
6	$(GTT)_6C$ / 53°C	16	$(TGC)_6C$ / 49°C
7	$(GTT)_6C$ / 55°C	17	$(TGC)_6C$ / 51°C
8	$(GTT)_6T$ / 45°C	18	$(TGC)_6C$ / 53°C
9	$(GTT)_6T$ / 47°C	19	$(TGC)_6C$ / 55°C
10	$(GTT)_6T$ / 49°C	20	Ladder

Figure A.16. Annealing Temperature (T_a) Screen #12. Diamonds denote optimum T_A.



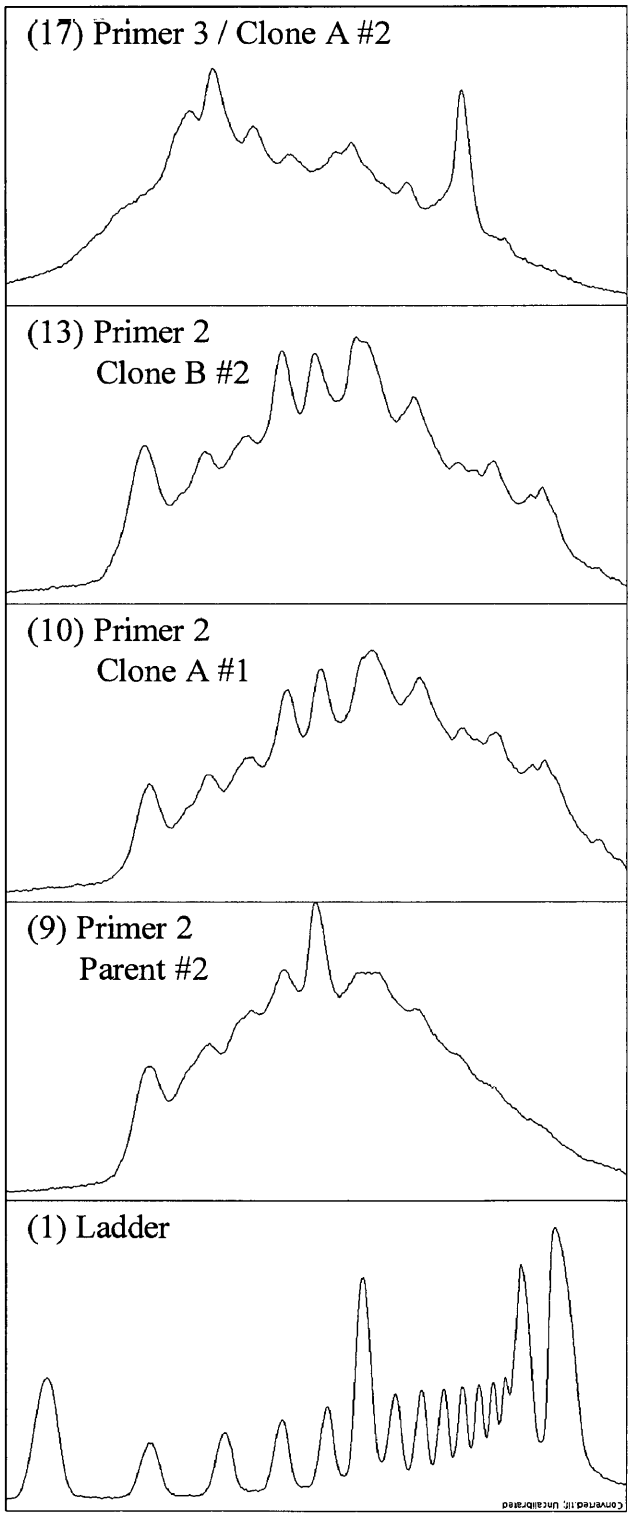
Lane	Primer / T_a	Lane	Primer / T_a
1	Ladder	11	$(TGC)_6G$ / 51°C
2	$(TGC)_6G$ / 45°C	12	$(TGC)_6G$ / 53°C
3	$(TGC)_6G$ / 47°C	13	$(TGC)_6G$ / 55°C
4	$(TGC)_6G$ / 49°C	14	Ladder

Figure A.17. ISSR of parent and two tissue culture offspring. Three primers are tested to verify reproducibility between DNA extractions and offspring. Tissue from Parent, Clone A, and Clone B was subjected to replicate DNA extractions (#1 and #2).



Lane	Primer / Source	Lane	Primer / Source
1	Ladder	11	2 / Clone A #2
2	1 / Parent #1	12	2 / Clone B #1
3	1 / Parent #2	13	2 / Clone B #2
4	1 / Clone A #1	14	3 / Parent #1
5	1 / Clone A #2	15	3 / Parent #1
6	1 / Clone B #1	16	3 / Clone A #1
7	1 / Clone B #2	17	3 / Clone A #2
8	2 / Parent #1	18	3 / Clone B #1
9	2 / Parent #2	19	3 / Clone B #2
10	2 / Clone A #1	20	Ladder

Figure A.18. Plots of lanes 1, 9, 10, 13, and 17 of ISSR from Figure 26. Length of fragments may be determined by interpolation based upon the molecular weights (bottom graph).



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