Molecular Analysis of Northern Red Oak *(Quercus rubra)* Populations from the Greater Sudbury Region: Genetic variation and Gene Expression

By

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Abstract

The main objectives of the present study were to 1) analyze genetic variability of *Q. rubra* populations from the mining region of Sudbury (Ontario) using RAPD marker system and 2) analyze the expression of nickel and copper resistance genes in *Q. rubra* populations. The level of polymorphic loci within populations was high ranging from 61 % to 72% despite a high level of gene flow (2.4) and the population differentiation (G_{ST}) value was low (0.17). All *Q. rubra* populations analyzed were genetically sustainable. Moreover, this study revealed that all populations were genetically closely related with genetic distance values varying from 0.17 to 0.35. A zinc finger protein of *Arabidopsis thaliana* (ZAT11) involved in nickel resistance was differentially expressed in samples analyzed. There was a 120x up-regulation of ZAT11 expression in samples from metal contaminated areas of Wahnapitae Dam compared to other sites. No association between soil metal levels and expression of ZAT11 was established.

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Table of Contents

List of Figures

List of Tables

Chapter 1: Literature Review

1.1.0 Mining in Sudbury, ON, Canada

In the late 1880's the discovery of an immense sulphide ore body in Sudbury, Ontario, Canada lead to the beginning of a world renowned mining, milling, smelting and refining industry (Freedman and Hutchinson 1980). This ore was composed of approximately 25% sulphide minerals including Iron (Fe), Copper (Cu) and Nickel (Ni) at a ratio of 6:1:1. Annual Production rates for Ni and Cu have continuously increased since the discovery and have presently reached 275,000 and 379,700 metric tonnes respectively, with a significant production rate of other metals including Gold (Au), Silver (Ag), Platinum (Pt) and Lead (Pd) (Winterhalder 1996).

The first step in processing the ore involved removing the sulphur in a process called roasting. This consisted in piling the ore on beds of cordwood to be continuously burnt in the open for as little as two months (Boldt 1967). The open roast yard technology was used between 1888 and 1929 and during this time, an estimated 10 million tonnes of sulphur dioxide (SO_2) was emitted into the surrounding atmosphere (Laroche, Sirois, and McIlveen 1979). This caused over 10,000 hectares of landscape in the Greater Sudbury Region (GSR) to be completely barren of vegetation and over 36,000 hectares of landscape to contain only herbaceous trees and shrubs (DeLestard 1967). The only trees found on the dry barren sites having survived the stresses responsible for the loss of vegetation were woody species including white birch *(Betula papyrifera),* red maple *(Acer rubrum)* and red oak *(Quercus rubra)* (Winterhalder 1996).

Gradually, more efficient smelting technologies were employed which released SO_2 and other mineral pollutants through three tall smokestacks located at Coniston, Copper Cliff and

Falconbridge smelters. Although more efficient, these smokestacks still released a significant amount of pollutants into the atmosphere (Freedman and Hutchinson 1980). In 1970, the 380m high Inco Limited "superstack" was constructed to disperse the sulphur gases and other by-products away from the city. At this time, this superstack was known as the world's greatest point source emitter of SO_2 (Summers and Whelpdale 1976). By 1994, strategies were implemented by Inco to decrease SO_2 emissions, like improvement of milling and flotation processes and recovery into liquid SO_2. This contributed to the reduction of emissions by 90% from the peak 1960 value of 265,000 tonnes a year (Winterhalder 1996).

1.1.1 Reclamation

The loss of vegetation induced by decades of mining activities caused soil erosion in the GSR (Winterhalder 1996). This eroded soil has been removed of a significant portion of its topsoil resulting in calcium (Ca), magnesium (Mg), manganese (Mn), nitrogen (N) and phosphorus (P) deficiencies (Smith 2008). Soils became acidic with pH ranging from 2.0 to 4.5 and metal content increased significantly within smelter proximity (Winterhalder 1996). For example, the average Cu and Ni content in organic horizons in distant reference sites normally range between 15-40 mg/kg and 20-30 mg/kg respectively, but reached 9,700 mg/kg and 6,960 mg/kg for Cu and Ni, respectively in sites surrounding smelters (Hazlett *et al*. 1983).

In 1969, the Sudbury Environmental Enhancement Program (SEEP), which included the Ontario Department of Lands & Forests and the Laurentian University Biology department, initiated land reclamation that planted several thousand trees in the GSR. Twenty-nine tree species were planted, half native to the area and the second half exotic. The mortality rate of the trees planted in Coniston soils was nearly 100%. While in Skead, there was a reasonable survival rate, but

growth rate was very poor. SEEP subsequently conducted research towards reclamation of Sudbury's landscape, more specifically, soil amendment. The primary discovery was that pH levels of Sudbury soils combined with elevated Cu and Ni levels were preventing the proper growth of planted trees (Hutchinson and Whitby 1972). SEEP researchers also discovered that liming the soils increased germination and growth of planted trees. This process involved the application of Ca and Mg rich materials to soil in various forms including marl, chalk, limestone or hydrated lime to neutralise soil acidity and increase activity of soil bacteria (Winterhalder 1996). Subsequent reclamation projects involved not only tree planting but pH analysis, liming and fertilizing of barren and semi-barren sites in the GSR (Hutchinson and Whitby 1972). Since 1978, over nine million trees and shrubs have been planted and over three thousand hectares of land have been limed, fertilized and seeded (VETAC 2015).

1.1.2 Metal and pH Analysis in Soil

Several studies have been conducted on metal accumulation in soils within the GSR and its effect on ecosystem health (Hutchinson and Whitby 1972; Nkongolo *et al*. 2013; Winterhalder 1996; Wren 2012). Current concentrations of total metals in populations located in close proximity to Sudbury smelters still exceed the acceptable limits set by the Ontario Ministry of Environment and Energy (OMEE) (Appendix 2) (Nkongolo *et al*. 2013; Wren 2012). Significant differences in mean concentrations of total aluminum (Al), arsenic (As), Cu and Ni have been observed in soils located within 15 kilometers of Sudbury smelters compared to reference populations. Mean concentration of bioavailable elements showed significant differences between contaminated and reference populations. Soil pH levels in Sudbury populations range from 2.35 to 6.7 and decrease the closer it lies in proximity to Sudbury smelters (Nkongolo *et al*.

2013; Tran *et al*., 2014). Limed populations have pH levels ranging from 4.12 and 6.7 which is significantly higher than populations that are not limed which range from 2.35 and 4.87 (Nkongolo *et al*. 2013).

1.2.0 Species of Interest

Kingdom: *Plantae*

Order: *Fagales*

Family: *Fagaceae*

Genus: *Quercus*

Section: *Lobatae*

Species: *Quercus rubra*

Description

Northern red oak (*Quercus rubra*), also known as common red oak, eastern red oak, mountain red oak, and gray oak is an economically and ecologically important forest tree, native to Eastern Northern America (Birchenko 2008). This tree ranges from 20 to 30 m in height and 61 to 91 cm in diameter and has a life expectancy of 150 to 250 years (Sanders 1980). Under optimal conditions, this fast growing tree can live up to 500 years. Leaves of the *Q. rubra* species are deciduous with an elliptical shape, ranging from 10 to 25 cm in length and 8 to 15 cm in width. The leaves are divided into 7-11 shallow wavy lobes and contain irregular bristle-tipped teeth. The upper half of the leaf is dull green gradually changing to a lighter green below (Appendix 1) (Neson 2003).

Distribution

Q. rubra has a wide geographical distribution which ranges from 60° to 96° West in longitude from Nebraska to the Atlantic coast and from 32° to 47° North in longitude from northern Ontario to southern Alabama. In eastern deciduous forests, *Q. rubra* is the most dominant oak species located on lower and north facing slopes (Alexander and Woeste 2014).

Establishment

Q. rubra grows on mesic slopes and well-drained uplands commonly facing north or east. For optimal growth, the tree should be fully exposed to the sun and growing in well-drained, slightly acidic, sandy soil. Acorns begin to produce when the tree reaches the age of 20-25 years; however they are not produced in abundance till they reach between 40 and 50 years of age. The germination of the acorns occur in the spring after over-wintering and breaks of dormancy (Neson 2003).

Uses

The wood of red oak trees is heavy, hard and close-grained, making it susceptible to a variety of finishes. For this reason, *Q. rubra* is an important source of hardwood lumber. This lumber is used for various wood products such as flooring, furniture, veneer, interior finishing cabinets and paneling. The tree is also useful for various species of birds and mammals both as shelter and nesting site. Their acorns are also used as a source of food for various mammals and birds both small and large (Rook 2006).

1.3.0 Measuring the Sustainability of *Q. rubra* Populations

Genetic variability can be defined as differences among individuals in a population at the genotypic level (Herron and Freeman 2014). A high degree of genetic variability present in a population is an indicator of adaptability and survival in varying environmental conditions. This allows the species to remain healthy, sustainable and to maintain a viable population size (Grant 2010).

Understanding the degree of genetic variation present within and among populations is of great ecologic and economic importance (Conner and Hartl 2004). Genetic variation within a population is often caused by mutations, recombination, migration, natural selection or random genetic drift. However, there is also strong evidence that changes in genetic variation can also occur very rapidly in plants in response to anthropogenic activities such as increased levels of metals (Nordal *et al*. 1999), herbicides (Heap 1997), ozone pollution, (Davidson and Reiling 1995), atmospheric carbon dioxide increases (Ward *et al*. 2000), and timber harvesting (Law and Salick 2005).

Genetic variation has been the subject of multiple studies. Reductions in genetic variability in response to metal contamination in *Deschampsia cespitosa* and *Armeria maritima* have been reported (Bush and Barrett 1993; Mehes-Smith and Nkongolo 2015; Vekemans *et al*. 1996). Other studies have found no significant difference among metal contaminated and reference populations of *Agrostis stolonifera* and *Arrhenatherum elatius* (Ducousso *et al*. 1990; Wu, Bradshaw, and Thurman 1975). Recent reports which utilized ISSR markers to analyze genetic variation in hardwood species including *B. papyrifera and Q. rubra* revealed moderate to high levels in both metal contaminated and reference populations of the GSR (Nkongolo *et al*. 2013;

Theriault, Nkongolo, and Michael 2014; Tran A *et al* 2014).

1.3.1 Molecular Markers

A genetic marker is an area on a chromosome that can be identified in the genome either by gel electrophoresis or by cytogenetic analysis (Semagn, Bjørnstad, and Ndjiondjop 2006). These markers can be generated by mutations or changes in genomic loci. Genetic markers offer a means to measure genetic diversity at a molecular level. Before choosing a molecular marker, certain desirable properties must be considered including high polymorphism, dominance or co-dominance, inheritance, accessibility, cost, reproducibility, and method of analysis such as hybridization based or polymerase chain reaction (PCR) based. Because there isn't a molecular marker which satisfies each of these properties, choosing one that meets most of the criteria based on the specific study is important (Semagn *et al*. 2006).

1.3.2 Hybridization-Based Molecular Markers

The first nucleic acid marker developed was the hybridization-based marker restriction fragment length polymorphisms (RFLP). An RFLP is the result of DNA mutations such as point mutations, deletions or insertions, translocations, inversions and duplication of a restriction site. This results in a gain, loss or relocation of a cleavage site. This technique involves digestion of DNA by restriction enzymes generating fragments of different sizes. The banding pattern is created using gel electrophoresis followed by southern blot hybridized with a probe labeled with either radioisotopes or non-radioisotopes. RFLPs are co-dominantly inherited, can estimate heterozygosity and are highly reproducible. However, a large amount of DNA is required, the technique often produces low level of polymorphism in certain species, few loci are detected per

assay and the technique is time consuming and costly (Smouse and Chakraborty 1986).

1.3.3 PCR-Based Markers

With the invention of the polymerase chain reaction (PCR), new approaches to the development of genetic markers have been established. They had several advantages compared to hybridization-based markers including: 1) small quantities of DNA required, 2) no need for radioisotopes for the majority of techniques, 3) some applications requiring no knowledge of sequences, 4) higher levels of polymorphism, 5) detection in a short period of time 6) low cost, and 7) simultaneous screening of many genes or loci (Wolfe and Liston 1998). There are two types of PCR-based markers: The first using site-specific primers such as single nucleotide polymorphism (SNP) and simple sequence repeats (SSR) requiring prior knowledge of the targeted sequence and the second using arbitrary or semi-arbitrary PCR primers such as inter simple sequence repeats (ISSR) and random amplified polymorphic DNA (RAPD) in which no prior knowledge of sequence is required

SNP markers detect differences at single nucleotides within a DNA sequence as a result of insertions or deletions. SNP are widely distributed throughout the genome of many organisms including plants. Their co-dominance, ability to work with extremely degraded DNA, high occurrence in the genome and possibility to multiplex hundreds or thousands on one chip have made them a popular choice for genetic variation studies (Balloux and Lugon-Moulin 2002).

SSR or microsatellites are widely distributed; short nucleotide sequences (2-9 bp) which are notable for their co-dominance and high allelic variation at each locus. SSR require amplification by PCR followed by separation of fragments using the gel electrophoresis technique. Studies

have reported that microsatellites are more variable than RAPD and therefore have the potential to show polymorphism in species otherwise characterized by low levels of genetic diversity. However, challenges often occur during the construction of enriched libraries and species-specific primers making the technique time consuming, labour intensive and costly (Jorgenson and Witte 2007).

ISSR primers are generated from arbitrary oligonucleotide sequences (15-22bp). The ISSR technique amplifies DNA fragments at various loci that are within an appropriate distance between two identical SSR motifs that are oppositely oriented. ISSR require amplification by PCR followed by separation of fragments using the gel electrophoresis technique. ISSR primers operate at a low cost, are highly reproducible due to longer primers and higher annealing temperatures and amplify only dominant markers (Godwin *et al*. 1997).

RAPD is a dominantly inherited marker, which relies on single arbitrary decanucleotide primers to amplify DNA fragments. RAPD loci are abundant, randomly dispersed throughout the genome and generate many polymorphic bands in coding and non-coding regions, as well as repeated or single-copy sequences covering the entire genome. In order to produce a RAPD product, amplification of DNA by PCR is required followed by separation of fragments using gel electrophoresis (Semagn *et al*. 2006; Williams *et al*. 2000). The RAPD technique is time effective compared to RFLP, which requires several days, cost effective and simple. RAPD generate universal primers which can be used for all species and small quantities of DNA are required (Fairbanks and Andersen 1996). However, precautions are needed to ensure that no cross-contamination occurs since these random primers are diverse. RAPD primers are

composed of 10 arbitrary oligonucleotides and are used under relaxed conditions, which can result in multiple priming sites with varying degrees of similarity. Also, the RAPD technique generates many fragments of similar mobility that originate from non-homologous regions and for this reason, it is more difficult to obtain clear and distinct bands between and within species (Sanchez de la Hoz *et al*. 1996). Despite these limitations, several studies replicating RAPD analyses with other types of molecular markers have proved its reliability and capacity of replicating consistent results with improved laboratory techniques and band scoring procedures (Fairbanks and Andersen 1996).

1.4.0 Molecular Mechanisms of Nickel Toxicity and Tolerance

The Function of Nickel in Plants

Ni is an essential micronutrient for plants. This metal is naturally present in soil at concentrations of ≤100 ppm and is present in plants at concentrations of 0.05-10 mg/kg dry weight (DW) (Chen *et al*. 2009). At the biological level, Ni functions in the production of viable seeds, and is also involved in plant growth, Fe absorption and N metabolism. At the molecular level, Ni is a cofactor for several enzymes including urease, a metalloenzyme which is responsible for metabolizing urea nitrogen into ammonia (Brown *et al*. 1987).

Uptake and Transport of Nickel in Plants

Uptake of Ni by plants is dependent on several factors including plant metabolism, soil acidity and the presence of other metals. Ni transport is primarily achieved through the root system from roots to shoots, and occasionally in leaves through the transpiration stream via the xylem. In its soluble form, Ni^{2+} absorption can be conducted via the cation transport system, competitively

with Cu^{2+} and Zinc (Zn^{2+}) or non-competitively from the Mg ion transport system. In the insoluble form, Ni is primarily absorbed in root cells by endocytosis. Proteins including high-affinity Ni transport proteins, metallothionein and metallochaperones have also been reported to be involved in secondary active transport of chelated Ni^{2+} (Chen *et al*. 2009).

Toxicity effects of Nickel on Plants

Nickel pollution, caused by anthropogenic activities including mining, vehicle emissions and fertilizer application have been known to cause soils to reach up to 30x their natural concentration of Ni (Chen *et al*. 2009). A common symptom of Ni toxicity includes leaf chlorosis and necrosis as a result of decreased Fe uptake and metabolism disruption. Toxicity symptoms also include inhibition of growth, disruption of photosynthesis, and retardation of germination (Bhalerao *et al*. 2015). These symptoms are strongly dependent on several factors including cultivation conditions, exposure time, growth stage, Ni concentration and plant species (Chen *et al*. 2009).

Toxicity Mechanisms of Nickel in Plants

Detoxification responses of Ni in plants have been extensively studied and are likely induced by indirect mechanisms including interference with other essential metal ions and induction of oxidative stress (Foyer and Shigeoka 2011). In excess concentrations, Ni inhibits the absorption of metals including Ca, Cu, Fe, Mg, Mn and Zn eventually leading to deficiencies. These metals are required for the function of several metalloenzymes including superoxide dismutase (SOD) and catalase (CAT). For this reason, uptake of toxic levels of Ni in plants can lead to a significant reduction in the biosynthesis of metalloenzymes. Additionally, Ni toxicity in plants

also interferes with their ability to scavenge reactive oxygen species (ROS) and to produce several antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-PX), glutathione reductase (GR), peroxidase (POD), glutathione peroxidase (GOPX) and ascorbate peroxidase (APX) (Chen *et al*. 2009). The accumulation of ROS leads to a process referred to as oxidative stress which causes chemical toxicity, damage to cellular components and eventually cell death (Foyer and Shigeoka 2011).

Mechanisms of resistance to nickel in plants

Resistance to toxic levels of metals in plants plays an important role in the survival and adaptation of a plant to its environment. Mechanisms of resistance of metals include avoidance and tolerance. The avoidance strategy involves the restriction of absorption of metals from soil into the plant root. Mechanisms of metal avoidance include immobilization by mycorrhizal association and metal sequestration by exuding organic compounds from root (Emamverdian *et al*. 2015). When the avoidance strategy is not achieved, tolerance mechanisms are activated.

Plants often develop tolerance mechanisms that involve biochemical and physiological adaptation to toxic concentrations of metal in soils. Genes that have been hypothesized to play a role in Ni tolerance include: glutathione reductase (GR), glutathione-s-transferase (GST), serine acetyltransferase (SAT), nicotianamine synthase (NAS3), metal transporter (NRAMP), 1-aminocyclopropane-1-carbozylic acid deaminase (ACC), high affinity nickel transporter family protein (AT2G16800), zinc transporter of Arabidopsis thaliana (ZAT11), iron-regulated protein (IREG), thioredoxin family protein and putative transmembrane protein (TMP).

Glutathione is a metal chelator that contains a thiol and carboxylic group allowing its formation with Ni and other metals. Once the complexes are formed, they are sequestered into vacuoles by ABC-type transporters (Viehweger 2014). In the Ni hyperaccumulator *Thlaspi goesingense,* increased levels of Ni in plants have been demonstrated to cause the over-expression of an enzyme called glutathione reductase (GR) involved in the maintenance of high levels of GSH. This increase in GSH results in an increased resistance to the growth inhibitory and oxidative stress induced effects of Ni (Freeman *et al*. 2004).

Glutathione-s-transferase (GST) is a family of enzymes that catalyze the conjugation of electrophilic substrates to reduce glutathione (GSH). The resulting complexes are transported to a vacuole for further processing or degradation. The removal of GSH facilitates the metabolism, sequestration or removal of xenobiotic in roots. Recent studies have discovered increases in GST activity in root and shoot tissues in response to Ni toxicity in wheat (*Triticum aestivum)*. More specifically, Ni was found to activate an isoform of glyoxalase I, which plays an important role in the degradation of methylglyoxal (MG). During stress, detoxification of MG occurs and GSH levels are reduced and regenerated (Gajewska and Skłodowska 2008).

High activity of serine acetyltransferase (SAT) is involved in Ni resistance in the Ni hyperaccumulator *T. goesingense*. SAT is responsible for catalyzing the acetylation of L-serine to produce O-acetyl-L-serine (OAS), a positive regulator of sulphur assimilation and an intermediate in the biosynthesis of the amino acid cysteine. The up-regulation of SAT in *T. goesingense* has been associated with this species ability to hyperaccumulate Ni and to resist damaging oxidative effects (Freeman *et al*. 2004).

Nicotianamine synthase (NAS3) is an enzyme responsible for the synthesis of Nicotianamine (NA) and plays a role in Ni tolerance in plants. Studies have revealed that exposure to Ni triggers accumulation of NA-Ni chelates in the xylem in the Ni hyperaccumulator *T. caerulescens* in a Ni dose-dependent manner. Once in the roots, Na bound to Ni is redirected to aerial parts from root-to-shoot (Mari *et al*. 2006).

The family of natural resistance-associated macrophage proteins (NRAMP) is a family of metal ion transporters, which are an integral part of the cell membrane. NRAMP proteins help with the transportation of metals including Mn^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Cadmium (Cd^{2+}), Ni^{2+} and Cobalt (Co^{2+}). Homologues of this gene family have been found in several organisms including bacteria, insects, mammals, plants and yeast (Williams *et al*. 2000). Mizuno *et al* (2005) have also established the role of NRAMP transporters in Ni^{2+} transportation and homeostasis.

1-aminocyclopropane-1-carboxylic acid deaminase (ACC) is an enzyme present in plant growth-promoting bacteria and is the biosynthetic precursor of the plant hormone ethylene. ACC has been demonstrated to lower plant ethylene levels and decrease the negative effects of various environmental stressors including Ni toxicity and contributing to increased Ni tolerance in canola (*B. napus*) (Stearns *et al*. 2005).

High affinity transporter family protein (AT2G16800) binds Ni and is involved in transportation of Ni across the plasma membrane in the *Arabidopsis thaliana* species (Stearns *et al*. 2005).

Zinc finger protein (ZAT11) is a C_2H_2 zinc finger protein of *A. thaliana* and a nuclear localized transcriptional regulator. ZAT11 positively regulates primary root growth at normal conditions and negatively regulates Ni^{2+} tolerance at excess levels of Ni. Mechanisms of reduced tolerance of Ni^{2+} by ZAT11 is believed to involve the repression of transcription of a vacuolar Ni^{2+} transporter gene (Liu *et al*. 2014).

The family of iron-regulated proteins (IREG) is involved in the transport of transition metals from long distances in the *A. thaliana* species. IREG genes have long been known to encode Fe transporters, however, recent studies have discovered that IREG has low substrate specificity. Thus, under Fe deficiency, accumulation of other transition metals including Ni occurs where the metal is then transported into the vacuole (Schaaf *et al*. 2006).

Thioredoxin reductase (Trr1) plays a vital role in the reduction-oxidation system of thioredoxin. Following oxidative stress, this enzyme catalyzes the reduction of thioredoxin by NADPH. For this reason, Trr1 plays a vital role in protection and detoxification of cells experiencing oxidative stress as a result of metal toxicity. More specifically, Trr1 plays an important role in cellular defense against Ni-induced DNA damage via a knockdown system of Trr1 using small interfering RNA (Kim and Seo 2012).

Putative transporter protein (TMP) has been established to be associated with Ni resistance during the transcription analysis of white birch (*Betula papyrifera)* (Theriault *et al*. 2016a). Its exact function is still unknown.

1.4.1 Molecular Mechanisms of Copper Toxicity and Tolerance

The Function of Copper in Plants

Copper (Cu) is an essential micronutrient required at very low concentrations for normal growth and development (Kobayashi *et al*. 2008). On average, plants maintain Cu levels at $10\mu g.g^{-1}$ (DW) but this differs significantly between plant species. Copper functions as a structural element in regulatory proteins, participates in photosynthetic electron transport, mitochondrial respiration, oxidative stress responses, cell wall metabolism and hormone signalling. This metal is also a cofactor in many enzymes including Cu/Zn superoxide dismutase (SOD), cytochrome c oxidase, amino oxidase, laccase, plastocyanin, and polyphenol oxidase. For this reason, Cu functions in signalling of transcription, protein trafficking machinery, oxidative phosphorylation and Fe mobilization (Yruela 2005).

Uptake and Transport of Copper in Plants

Uptake of Cu in plants is primarily achieved from the root system, from roots to shoots via the xylem using complexing ligands. Although little is known about the specific uptake of Cu, there has been evidence that it is accomplished competitively with Fe and strongly associated with organic matter (Ryan *et al*. 2013). Several families of metal transporters have been identified that are involved in Cu transportation including 1) P-type ATPase Cu-transporters, which use ATP to pump a variety of charged substrates including Cu^{2+} across biological membranes; 2) Cu chaperones, belonging to a family of cytosolic, soluble, low-molecular-weight metal-receptor proteins named metallochaperones, involved in intracellular transportation of Cu (Liao 2000).

Toxicity effects of Copper on Plants

Toxic levels of Cu in soils can occur either naturally or as a result of anthropogenic activities

including mining, smelting and waste disposal technologies. Toxicity effects of Cu occur for most plant species at concentration higher than 20-50 mg kg^{-1} (DW) (Liao 2000). Toxic levels of Cu interfere with several cellular processes including photosynthesis. More specifically, Cu was shown to interfere with the biosynthesis of photosynthetic machinery modifying the pigment and protein composition of photosynthetic membranes. Lipid peroxidation, decreasing of lipid content and alterations in fatty acid composition of thylakoid membranes are also observed in Cu toxicity resulting in PSII membrane fluidity alterations. In terms of plant morphology, toxic levels of Cu cause inhibition of growth, reduced biomass and chlorosis (Küpper *et al*. 2009).

Toxicity Mechanisms of Copper in Plants

Toxic levels of Cu in plants are well known to cause oxidative stress from increased production of oxygen free radicals. More specifically, Cu catalyzes the formation of hydroxyl radicals (OH^-) inducing changes in antioxidative pathways including ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), superoxide dismutase (SOD), and guiacol peroxidase. These antioxidant responses are dependent on Cu concentrations and exposure time. The main target of Cu transport has been discovered to be the photosystem II (PSII). Both the acceptor and the donor side are highly sensitive to the toxic action of Cu and this action is highly dependent on Cu concentrations (Liao 2000).

Mechanisms of resistance to copper in plants

Cu resistance mechanisms include avoidance, tolerance and hyperaccumulation. Avoidance mechanisms of Cu toxicity in plants involve the binding of organic acids excreted by the plant by

certain mycorrhizal species, inhibiting metal uptake (Van Tichelen *et al*. 2001).

Different uptake mechanisms in plants are directly related to different mechanisms of metal tolerance at the whole plant level (Baker *et al*. 2001). If Cu successfully enters root cells, metal-binding proteins allow their transportation from roots-to-shoots. In excess, Cu is often transported to the vacuole, apoplast or to specialized cells (epidermal cells, trichomes) for storage to avoid toxicity (Liao 2000). A second tolerance mechanism of Cu in plants involves the accumulation of organic acids in the xylem, facilitating metal complexing and enhancing the mobility of Cu in the xylem and the chemical gradient for metal ions (Liao 2000). Genes that have been hypothesized to play a role in Cu tolerance include copper-transporting ATPase (RAN1), multi-drug resistance associated protein (MRP4), copper transporter protein (COPT1), and metallothionein (MT2B).

Copper transporting ATPase (RAN1): belongs to the P-type ATPase Cu-transporters and is classified as the subtype 1B ATPase. RAN1 is involved in intracellular transportation of Cu to chloroplasts and is localized at the post-Golgi compartment (Liao 2000). RAN1 is also involved in the delivery of Cu to ethylene receptors in *A. thaliana,* a hormone which plays a crucial role in plant growth (Yruela 2005).

Multi-drug resistance associated protein (MRP4) belongs to the subfamily of ATP-binding cassette (ABC) transporters, which are ATP-dependent and an integral part of plant detoxification. MRP4 has been demonstrated to be a multi-purpose membrane protein, suggested to be involved in vacuolar sequestration of potentially toxic metabolites and highly up-regulated

in roots and shoots of Cu-tolerant *B. pendula* (Keinänen *et al*. 2007).

Metallothioneins (MT2B) are cysteine-rich proteins that bind metals including Cd, Cu and Zn. MT's protect plants from oxidative stress by detoxifying hydroxyl radicals. MT2B is proposed to be involved in the distribution of Cu via the phloem and have a role in Cu tolerance, homeostasis and long distance transport of Cu in *A. thaliana* (Guo *et al*. 2008).

Copper transporter protein (COPT1) belongs to a family of putative copper transporters (COPT1-COPT5) in *A. thaliana* and is involved in the uptake and accumulation of Cu via the root apical zone. It is expressed in root tips and pollen grains and functions in root elongation and pollen development. In leaves, COPT1 is expressed exclusively in trichomes and stomatal guard cells, suggesting a role in Cu detoxification (Sancenon *et al*. 2004).

1.4.2 Measuring Gene Expression Levels in Plants

Several approaches can be used to analyze gene expression levels in plants. Targeted gene expression analysis can be conducted if the experimental approach is limited to specific genes or areas of the transcriptome. However, whole transcriptome sequencing has become more and more common and can be conducted in species with known transcriptome sequences. Common gene expression applications include Northern blotting, in situ-hybridization and real-time quantitative PCR as well as higher plex techniques such as microarrays and RNA-seq. Before choosing a technique several factors must be considered including the availability of fresh tissue, expected expression levels, and the pattern of candidate genes (Kramer 2005).

Northern blotting detects mRNA to analyze gene expression levels. Cells are first exposed to an enzyme that breaks down cell membranes and releases genetic material in the cells called protease. The mRNA is then separated from other cellular components and gel electrophoresis is used to separate different fragments of mRNA. The mRNA is then placed on a filter or other support using a technique called blotting. In order to identify the gene, the mRNA is incubated with a probe (short piece of complementary single-stranded DNA or RNA labelled with a radioactive molecule). The filter is finally placed against x-ray film to expose the probe, which can vary in intensity depending on how strongly the gene is expressed (Krumlauf 1992).

The *in situ* hybridization technique uses labeled probes to detect DNA or RNA sequences in a section of tissue (in situ) or in the entire tissue (ex: plant seeds, whole mount). The probe can either be radioactive or non-radioactive (Kramer 2005).

Quantitative reverse transcription PCR (RT-q-PCR) is used when the starting material is RNA. In this method, RNA is reverse transcribed into cDNA, which is subsequently used as the template for the PCR reaction. Absolute or relative quantitation can be used to calculate the result. In absolute quantitation, unknown samples are quantitated using a standard curve. A relative quantitation assay is used in gene expression analyses where changes in expression are compared to reference samples, often using housekeeping genes (Heid *et al*. 1996).

Microarrays are often applied to large-scale gene expression studies. Initially, microarrays were developed to study gene expression in populations of RNA but recently microarray protocols have been altered to analyze expression at the DNA and protein level as well. Microarray

technologies use an impermeable solid support such as glass, silicon chips or nylon membrane containing thousands of spots each representing a single gene potentially representing the entire genome of an organism. Microarray technology requires hybridisation, the process of joining two complementary strands of DNA to form a double stranded molecule (Babu 2004; Russo *et al*. 2003).

RNA-seq is a RNA profiling technique based on next-generation sequencing. This technique presents a method of characterizing and quantifying all RNA sequences present in a sample at high resolution. RNA-seq allows the reconstruction of known and novel transcripts at a single-base level, has a broad dynamic range and high level of reproducibility (Finotello and Di Camillo 2015; Wang *et al*. 2009).

1.4.3 Objectives

The objectives of the study were to 1) determine the level of genetic variation in *Q. rubra* populations from mining damaged ecosystems using RAPD marker system and 2) assess the level of expression of genes associated with nickel and copper resistance in *Q. rubra*.

Chapter 2: Analyzing the Genetic Variability of Red Oak *(Quercus rubra)* Populations From Metal Contaminated and Uncontaminated Populations in the Greater Sudbury Region (Ontario, Canada)

2.1.0 Introduction

Understanding the genetic variability of a population is crucial to gain a deeper understanding of the species survival and adaptation abilities in changing environments. Decades of mining activities in the Greater Sudbury Region (GSR) have caused significant damage on surrounding soil and vegetation. Periodical assessment of terrestrial ecosystems within this region is essential to determine long-term effects of restoration processes that often occur over an extended period of time.

The fitness and viability of *Q. rubra* populations planted, as part of regreening projects in the GSR has been the subject of many studies. The sustainability of *Q. rubra* from limed and unlimed areas in the GSR using intersimple sequence repeat (ISSR) markers has recently been studied in which, moderate to high genetic variability was reported. However, no significant difference in genetic variation among limed versus unlimed and metal contaminated versus uncontaminated populations was found (Tran *et al*. 2014).

RAPD and ISSR marker systems have both demonstrated to be equally reliable methods for the detection of polymorphism. Most often, ISSR primers detect higher polymorphism than RAPD primers because of the high level of variability in microsatellite loci. The discrepancy between variations revealed between RAPD and ISSR results from different targeted genomic areas which undergo a different evolutionary process due to selection forces (Qian *et al*. 2001). The

RAPD technique can detect more unique fragments between and within species than the ISSR technique. The specific objective of this study was to determine the level of genetic variation in *Q. rubra* populations from mining damaged ecosystems in the GSR using a RAPD marker system.

2.2.0 Materials and Methods

2.2.1 Sampling

Q. rubra leaf samples were collected from ten populations around the GSR. Twenty trees from each targeted population were selected for the study. The populations included seven metal contaminated sites located within 15km from Sudbury smelters (Airport, Daisy Lake, Falconbridge, Kingsway, Kukagami, Laurentian and Wahnapitae Hydro Dam,) as well as, three reference populations located as far as 100km away from Sudbury smelters (Capreol, St-Charles, and Onaping falls) (Figure 2.0). Leaves were collected if they possessed an elliptical shape with 7-11 shallow waxy lobes, wrapped in aluminum foil, immersed in liquid nitrogen and stored at -20°C until DNA extraction (Tran 2013).

2.2.2 Molecular Analysis

DNA Extraction

Genomic DNA was extracted from frozen leaf material using the CTAB extraction protocol as described by Mehes-Smith *et al*. (2007). The protocol is a modification of Doyle and Doyle (1987) procedure, modifications included the addition of 1% polyvinyl pyrrolidone (PVP) and 0.2% β-mercaptoethanol to the cetyl trimethylammonium bromide (CTAB) buffer solution, two additional chloroform centrifugation steps of ten minutes prior to the isopropanol spin and no addition of RNAse. After extraction, DNA was stored in the freezer at -20°C.

DNA samples were tested for quality and intactness by gel electrophoresis on 1% agarose gels in 0.5x Tris-Borate-EDTA (TBE) and pre-stained with 1μL of ethidium bromide. A mixture of 5μL of stock DNA and 1μL of 6x loading buffer was loaded into the wells of the agarose gel and was run at 60V for 90 minutes. Gels were then visualized under an ultra-violet light source, documented with Bio-Rad ChemiDoc XRS™ system and analyzed with Image Lab Software™.

DNA quantitation was performed using the Bio-Rad™ Quantitation Kit (catalogue # 170-2480). Concentrations were determined by fluorochrome Hoechst. The dye mixture contained 3.2mL of 1xTEN assay buffer, 6μL of 4 2 μg/mL of Hoechst dye and 28.79mL of ddH_2O. A standard curve was produced using known calf thymus DNA. For calf thymus concentration of 100ng/μL the following volumes were added: 1750ng, 1500ng, 1250ng, 1000ng, 750ng, 500ng, 250ng. For calf thymus concentration of 10 ng/μL the following volumes were added: 100ng, 75ng, 50ng, 25ng. Extracted DNA (2 μL) was added to the plates in duplicates. The DNA fluorescence intensity was measured using BMG LABTECH FLUOstar OPTIMA microplate multi-detection reader in fluorescence detection mode. The DNA concentration was standardized at 5 ng/μL.

RAPD Analysis

A total of 56 RAPD primers were pre-screened for polymorphism and reproducibility (Appendix 3). Amongst these primers, six were identified which produced strong bands and were subsequently used for RAPD analysis. They include: UBC 186, UBC 403, OPA 16, OPA 19, OPB 05 and OPB 17 (Table 2.0).

PCR amplification was carried out as described by Mehes-Smith *et al* (2007) with modifications. A 25µL total volume master mix was used with: 8.9µL distilled H_2O, 5µL $MgSO_4$, 2.1µL 10x buffer, 0.5µL dNTPs (equal parts dTTP, dATP, dCTP, dGTP), 0.5µL RAPD primer, a taq mix consisting of 3.475µL distilled H_2O, 0.4µL 10x buffer and 0.125µL Taq polymerase (Applied Biosystems) and 4µL of standardized DNA (4µL of distilled H_2O as a negative control). A drop of mineral oil was then added to the tube for the prevention of evaporation.

Amplification was conducted with the Eppendorf Mastercycler gradient. The amplification process including 1) Initial denaturation step at 95°C for 5 minutes; 2) 85°C for 2 minutes 3) Addition of *Taq;* 4) denaturation step at 95°C for 90 seconds 5) annealing step at 55°C for 2 minutes; 6) extension step at 72°C for 1 minute; (Repetition of steps 4-6 for 42 cycles); 7) final extension step at 72°C for 7 minutes. All 200 samples were amplified in duplicate to ensure reproducibility. After amplification, the samples were removed and placed in -20°C until further analysis.

DNA was separated on a 2% agarose gel in 0.5x TBE and pre-stained with 1µL of ethidium bromide. A mixture of 5µL of 6x loading buffer and 5µL of stock DNA was loaded into the wells of the agarose gel and was run at 64V for 2 hours. Gels were then visualized under an ultra-violet light source, documented with the Bio-Rad ChemiDoc XRS™ system and analyzed with Image Lab Software™.

2.2.3 Statistical Analysis

Due to the dominant nature of the marker system chosen for this study, the amplified RAPD bands were scored manually as either present (1) or absent (0). For a band to be present, it had to

appear in both duplicate amplification images on the Image Lab Software™ to ensure reproducibility. Popgene software version 1.32 (Yeh *et al*. 1997) was used to determine statistical parameters including the percentage of polymorphic loci, observed (Na) and expected (Ne) number of alleles, Nei's gene diversity (h) and Shannon's information index (I). The Free Tree Program version 1.50 was also used to calculate genetic distances using Jaccard's similarity coefficients. Using the similarity coefficients, a dendogram was constructed under the principle of minimum evolution using the neighbour-joining method (Saitou and Nei 1987).

2.3.0 Results

2.3.1 RAPD-PCR Amplification and Polymorphism

Detailed description of the six most polymorphic primers were selected to amplify all standardized DNA samples from the ten populations are represented Table 2.0 and amplified products are depicted in figures 2.1-2.6. The total number of bands generated by each primer varied from 21 t o 28. Band sizes ranged from 205bp to 2000bp.

2.3.2 Genetic Variability

Genetic variability was determined for the ten *Q. rubra* populations using the Popgene Software version 1.32 (Yeh *et al*. 1997) (Table 2.1). The percentage of polymorphic loci (%), the observed number of alleles (Na), the expected number of alleles (Ne), Nei's gene diversity (h) and Shannon's information index (I) are described in table 2.1. The mean values for Na, Ne, h and I were 1.69, 1.30, 0.19 and 0.29 respectively.

The total gene diversity (H_T) and the mean gene diversity among populations (H_S) were 0.2239 and 0.18552 respectively. The population differentiation (G_{ST}) value was 0.1728 (17.28%) and

the estimated gene flow (Nm) was 2.4. The levels of genetic variation in all populations were considered high as it varied from 61.07% (Capreol) to 71.81% (Daisy Lake) with a mean of 68.52%.

The observed number of alleles ranged from 1.6107 (Capreol) to 1.7181 (Daisy Lake) with a mean of 1.69, the expected number of alleles from 1.3635 (Falconbridge) to 1.2652 (Airport) with a mean of 1.30, Nei's gene diversity (h) from 0.1616 (Airport) to 0.2202 (Falconbridge) with a mean of 0.19, and Shannon's information index from between 0.2535 to 0.3373 with a mean of 0.29 (table 2.1).

2.3.3 Genetic Relationship

All markers were scored based on the presence or absence of amplification products observed as bands on the agarose gels. This data was then used to calculate genetic distances for the ten populations (table 2.2). The values were based on a scale ranging from 0 (identical) to 1 (different for all criteria) and the genetic distance values ranged from 0.2032 (Daisy Lake and Wahnapitae Hydro Dam) to 0.34921 (Falconbridge and Capreol). This dataset was used to create a dendogram to demonstrate the genetic relationships among the populations (Figure 2.7).

2.4.0 Discussion

Genetic Variation, Gene Flow and Population Differentiation

Analysis of soil chemistry from the targeted sites has been described in detail in Nkongolo *et al*. (2013). The focus of the present study was on genetic analysis using a RAPD marker system. A gene is defined as polymorphic if the frequency of one of its alleles is less than or equal to 0.99. There was no significant differences between metal contaminated and reference population in

terms of the level of polymorphism.

The observed number of alleles (Na) and the expected number of alleles (Ne) were calculated. Na measures the number of alleles based on the raw data obtained by RAPD analysis and it ranges from 0 to 2. Ne estimates the expected number of alleles that should be observed within the populations using the value of Na and it ranges from 0 and 2. Low values of Na and Ne lying closer to zero indicate homozygosity. Higher values of Na compared to Ne indicate heterozygosity (Cornuet and Luikart 1996). The mean value of Na and Ne were 1.69 and 1.30 respectively. These values could suggest high heterozygosity within the populations studied, which could be caused by factors including gene flow rather than inbreeding within populations.

Nei's gene diversity (h) is a parameter that measures the probability that, at a single locus any two alleles chosen at random from the population are different from one another. This is an estimate of the extent of genetic variability within a population and is measured on a scale of 0 to 1. Values closer to 0 indicate that the population is monomorphic with no allelic differences and values approaching 1 indicate a polymorphic population (Nei 1973). The mean value of h for the *Q. rubra* populations studied was 0.1869. This low value indicates low allelic differences within populations. Based on Nei's gene diversity, the *Q. rubra* populations chosen for this study are monomorphic with alleles of equal frequencies occurring in more, or all loci.

Shannon's information index (I) was calculated to determine the abundance and distribution of alleles within populations on a scale of 0 to 1. Values closer to 0 indicate an uneven distribution of alleles and values closer to 1 indicate an even distribution of alleles and an abundance of

species among the populations (Morris *et al*. 2014). I was calculated in each *Q. rubra* population and the mean was 0.29. This value indicates that there is an uneven distribution of alleles in these populations and low phenotypic diversity.

The total gene diversity (H_T) and the mean gene diversity within populations (H_S) were 0.2239 and 0.1852, respectively. H_T and H_S were used to calculate the coefficient of inter-population differentiation (G_{ST}) and was estimated using the equation [$G_{ST} = (H_T-H_S)/H_T$] (Nei 1973). G_{ST} provides a measure of proportion of gene diversity that is distributed among populations while also taking into account the uniformity and diversity within populations (Madhusudhana and Rajendrakumar 2015). It has been suggested that a value of G_{ST} between 0 and 0.05 indicates little genetic variation; between 0.05 and 0.15 indicates moderate differentiation, between 0.15 and 0.25 indicates great differentiation, and greater than 0.25 indicates very high genetic differentiation (Balloux and Lugon-Moulin 2002; Hartl and Clark 1997; Wright 1978). In the populations of *Q. rubra* used in this study, G_{ST} was 0.1728 (17.28%). This means that only 17.28% of total heterozygosity is accounted for by the differences among populations.

A contributing factor to genetic differentiation in the *Q. rubra* populations studied could be the potentially capacity for dispersal among fragmented populations by pollen and seeds. Although the bulk of pollen is generally deposited near the source plant, several studies have documented that *Q. rubra* is a wind-pollinated tree, generating vast amounts of pollen to ensure a sufficient level of fertilization of female flowers over receptor regions (Schwarzmann and Gerhold 1991). Pollen grains of the *Q. rubra* species are small and light which facilitates the transportation of a substantial fraction of released material over thousands of kilometers under suitable weather

conditions (Sofiev *et al*. 2006).

Gene flow (N_m), a parameter, which calculates the transfer of alleles from one population to another, was 2.394. The high gene flow in this study is likely a combination of pollen dispersal and seed distribution through animal dispersers. Also, the *Q. rubra* populations for this study were selected based on different wind directions which may have had an effect on introgression of alleles among populations and contributed to low population differentiation.

The genetic distance between two populations is described as the proportion of alleles that the two populations do not share (Nei 1973). Values of the distance matrix can vary between 0 and 1, a value of 0 indicating that the populations in questions are identical and a value of 1 indicating that the two populations contain no alleles in common. The genetic distances in this present study ranged from 0.2032 and 0.3492, which is indicative of close genetic relatedness of *Q. rubra* populations analyzed.

A dendogram was constructed using the neighbour-joining method. It showed with a high degree of confidence two main clusters, one including Daisy Lake, Wahnapitae Hydro Dam, and Laurentian and the second grouping all other populations (Figure 2.7)

Genetic Variation and Metal Contamination

The study revealed no significant differences in genetic variation among metal contaminated and reference populations of *Q. rubra,* despite high levels of total Ni (1,600 mg/kg) and Cu (1,313 mg/kg) concentrations (Nkongolo *et al*. 2013). This lack of differentiation among populations

could be attributed to low levels of bioavailable Ni (5.16 mg/kg) and Cu (9.13 mg /kg) (Nkongolo *et al*. 2013). It is important to note that the *Q. rubra* populations analyzed in this study represent the second generation of trees that survived metal contamination for several decades. For this deciduous species, two generations might be too short for any meaningful changes in the total DNA. Furthermore, plants possess homeostatic cellular mechanisms to regulate the concentration of metal ions inside their cells and to minimize damage caused by metal toxicity (Benavides, Gallego, and Tomaro 2005). The understanding of specific metal resistance mechanisms in the *Q. rubra* species as well as most species within the *Plantae* kingdom remains very limited.

Figure 2.0: Locations of targeted Q. rubra populations within the Greater Sudbury Region in Northern Ontario. 1: Daisy Lake; 2: Wahnapitae Hydro Dam; 3: Laurentian; 4: Kukagami; 5: Kingsway; 6: Falconbridge; 7: Capreol; 8: St-Charles; 9: Onaping Falls; 10: Airport; 11: Kelly Lake

Table 2.0: The nucleotide sequences of RAPD primers used to amplify DNA from *Q. rubra* population samples

RAPD Primers	**5' → 3'**	**GC content**	**Number of bands**	**Size (bp)**
UBC 186	GTGCGTCGCT	70%	27	205 - 1950
UBC 402	CCCGCCGTTG	80%	21	330-2000
OPA 16	AGCCAGCGAA	60%	23	230-2000
OPA 19	CAAACGTCGG	60%	25	230-2000
OPB 05	AGGGGTCTTG	60%	28	140-1900
OPT 17	CCAACGTCGT	60%	25	275-1900

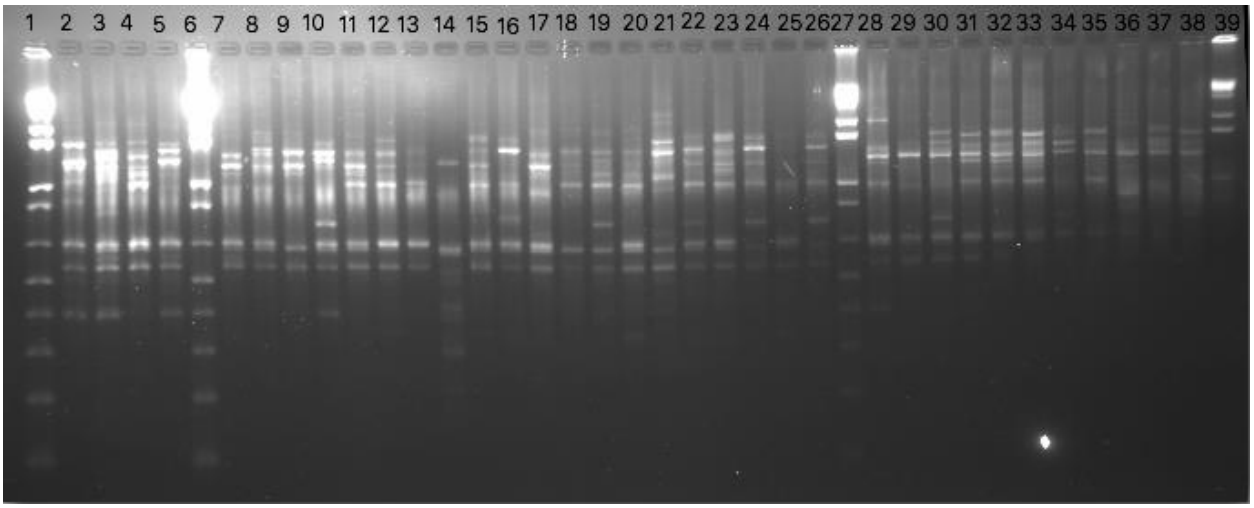

Figure 2.1: RAPD amplification of Q. rubra DNA samples with primer OPA16. Lanes 1, 6 and 27 contain a 1kb+ ladder; Lanes 2-5 contain Q. rubra samples from the Falconbridge population; Lanes 7-26 contain Q. rubra samples from the Capreol population; Lane 28-38 contain Q. rubra samples from the St-Charles population

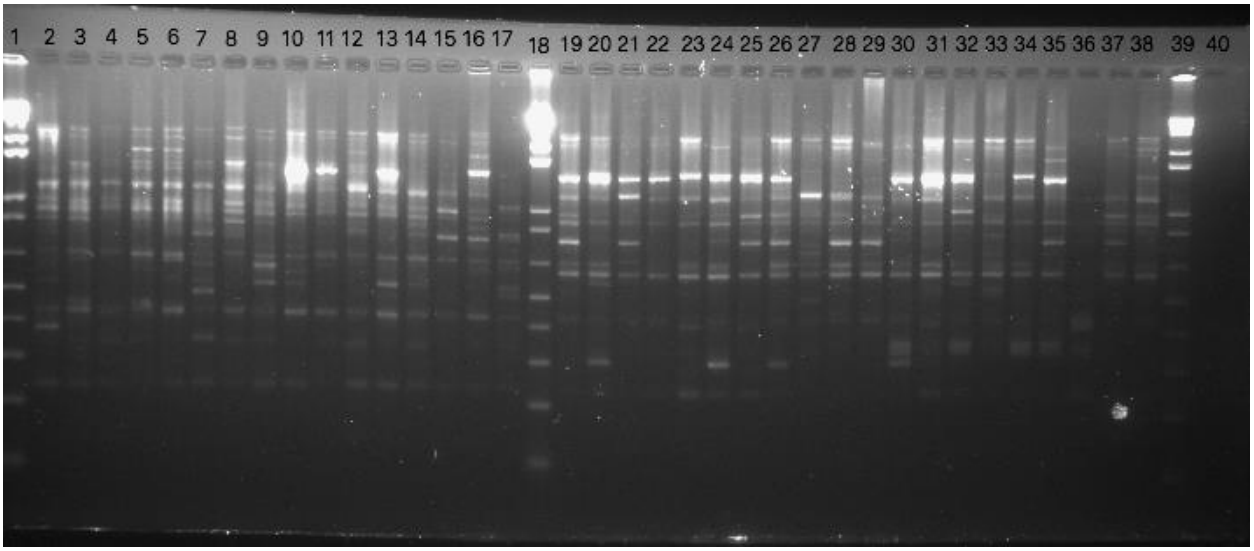

Figure 2.2: RAPD amplification of Q. rubra DNA samples with primer OPA19. Lanes 1, 18 and 39 contain a 1kb+ ladder; Lanes 2-17 contain Q. rubra samples from the Wahnapitae Hydro Dam population; Lanes 19-38 contains Q. rubra samples from the Laurentian population

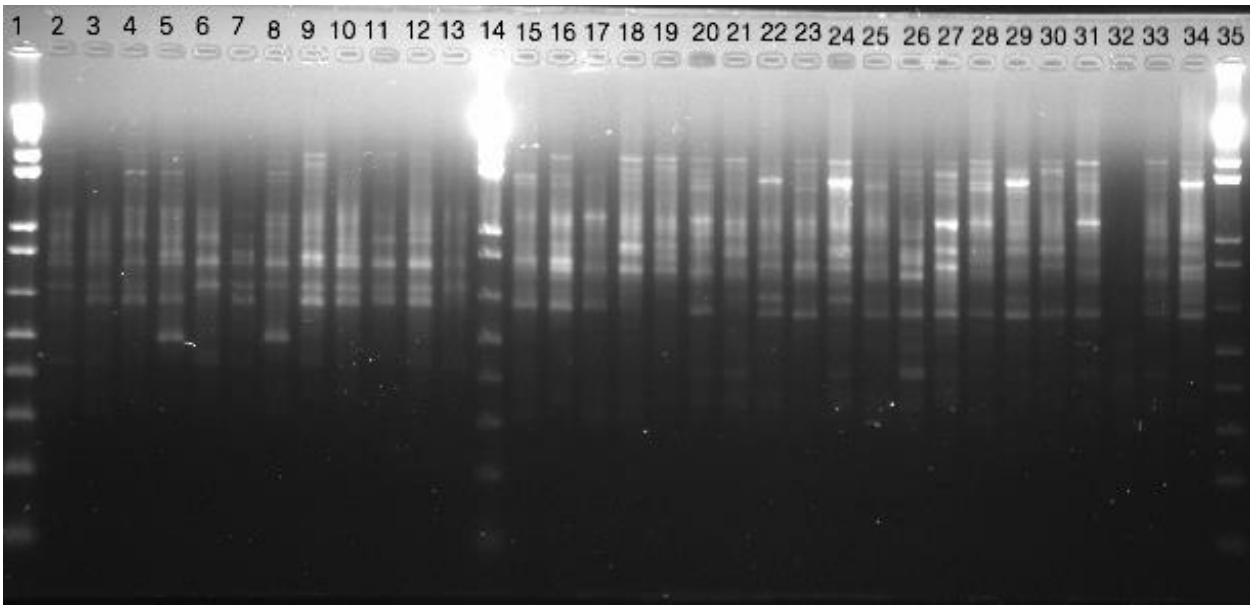

Figure 2.3: RAPD amplification of Q. rubra DNA samples with primer OPB05. Lanes 1, 14 and 35 contain a 1kb+ ladder; Lanes 2-14 contain Q. rubra samples from the Laurentian population; Lanes 15-34 contain Q. rubra samples from the Kukagami population

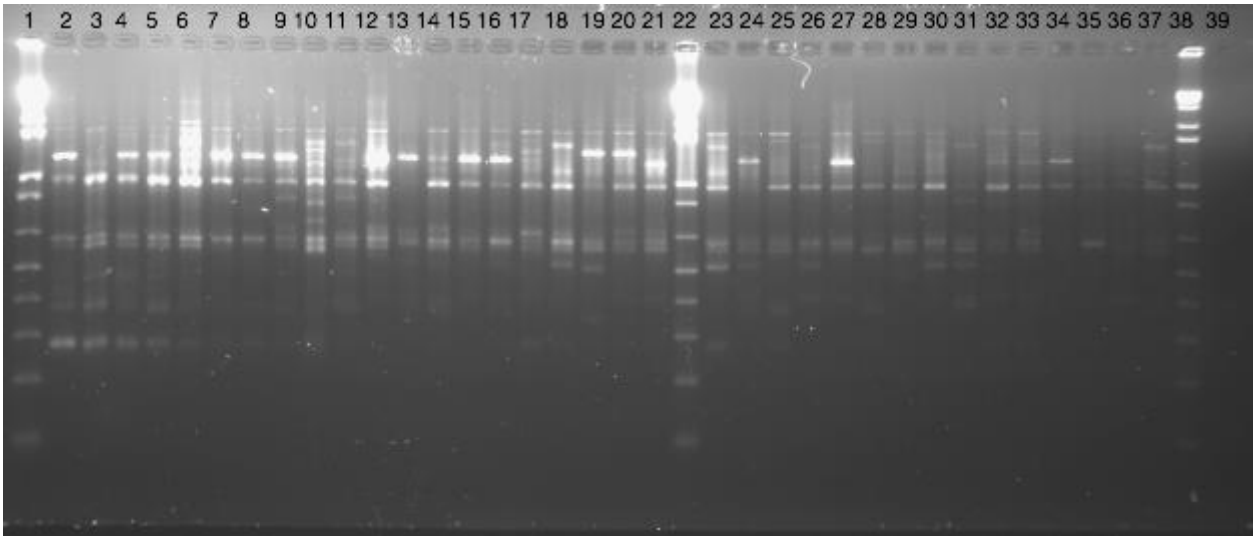

Figure 2.4: RAPD amplification of Q. rubra DNA samples with primer OPT17. Lanes 1, 22 and 38 contain a 1kb+ ladder; Lanes 2-21 contain Q. rubra samples from the Kingsway population; Lanes 23-37 contain Q. rubra samples from the Falconbridge population

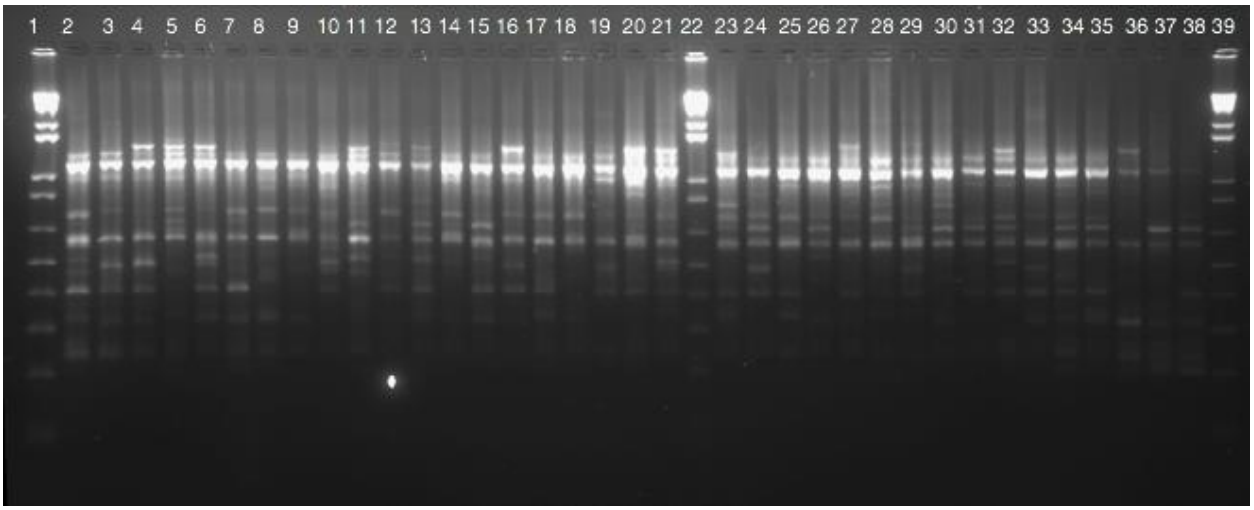

Figure 2.5: RAPD amplification of Q. rubra DNA samples with primer UBC186. Lanes 1, 22 and 37 contain a 1kb+ ladder; Lanes 2-21 contain Q. rubra samples from the Kingsway population; Lanes 23-38 contain Q. rubra samples from the Falconbridge population

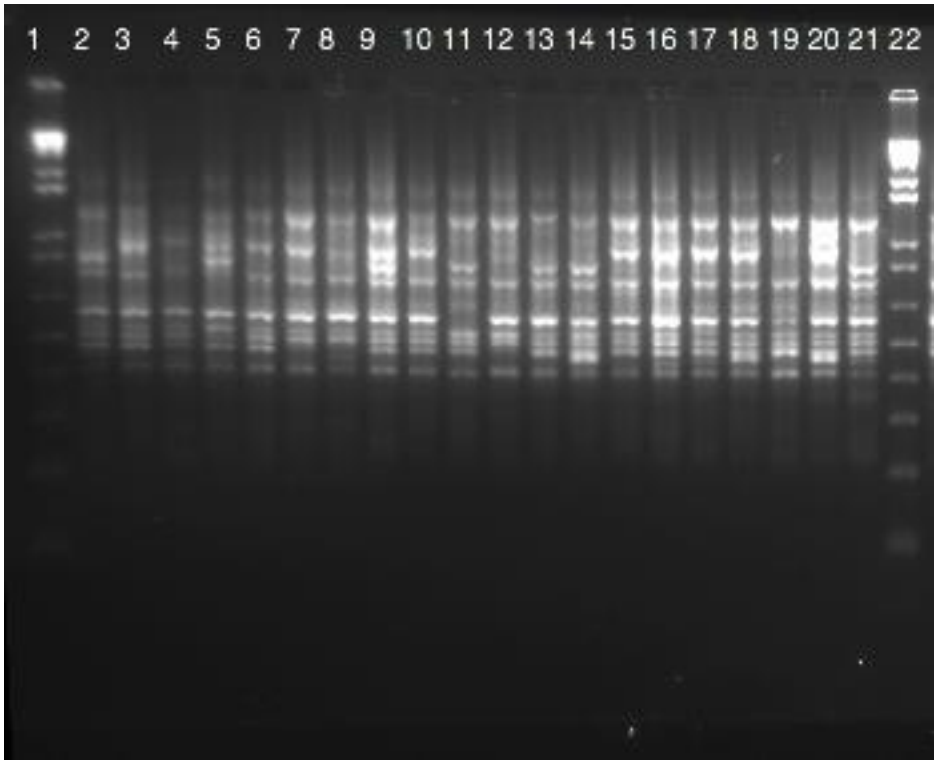

Figure 2.6: RAPD amplification of Q. rubra DNA samples with primer UBC402. Lanes 1, 22 contain a 1kb+ ladder; Lanes 2-21 contain Q. rubra samples from the Wahnapitae Hydro dam population

Table 2.1: Genetic Diversity parameters of *Q. rubra* populations based on RAPD data

Population	P (%)	Na	Ne	H	I
Daisy Lake	71.81	1.7181	1.3024	0.1859	0.2923
Wahnapitae	67.79	1.6779	1.2889	0.1787	0.2808
Hydro Dam					
Falconbridge	71.14	1.7114	1.3635	0.2202	0.3373
Laurentian	71.14	1.7114	1.3088	0.1898	0.2970
Kukagami	70.47	1.7047	1.2817	0.1754	0.2781
Kingsway	69.80	1.6980	1.3215	0.1963	0.3046
Airport	62.42	1.6242	1.2652	0.1616	0.2535
Capreol	61.07	1.6107	1.2817	0.1701	0.2636
St.Charles	68.46	1.6846	1.3093	0.1875	0.2912
Onaping Falls	71.14	1.7114	1.3034	0.1869	0.2929
Mean		1.69	1.30	0.19	0.29
		Ht	**Hs**	**Gst**	**Nm**
Overall		0.2239	0.1852	0.1728	2.3941

Genetic diversity descriptive statistics; P: percentage of polymorphic loci; Na: observed number of alleles; Ne: expected number of alleles; h: gene diversity (Nei, 1973); I: Shannon's information index; Ht: Total genetic diversity; Hs: Genetic diversity within populations; Gst: Genetic differentiation; Nm: Gene Flow

Table 2.2: Distance matrix generated from RAPD data using Jaccard's similarity coefficient analysis for 10 *Q. rubra* populations (Free Tree Program version 1.50)

	Daisy Lake	**Dam**	**Laurentian**	**Kukagami**	**Kingsway**	**Falconbridge**	**Capreol**	**St.Charles**	**Onaping Falls**	**Airport**
Daisy Lake		0.20325	0.25781	0.26923	0.26923	0.24806	0.28455	0.23200	0.30827	0.27200
Dam			0.24390	0.25600	0.32308	0.24800	0.27119	0.24590	0.29688	0.31452
Laurentian				0.29457	0.34586	0.27344	0.33871	0.28571	0.34586	0.29839
Kukagami					0.30534	0.28462	0.32258	0.25600	0.29231	0.34884
Kingsway						0.24409	0.34921	0.25600	0.34328	0.33594
Falconbridge							0.27273	0.17500	0.31061	0.34109
Capreol								0.25641	0.33600	0.35537
St.Charles									0.26984	0.30081
Onaping Falls										0.30952
Airport										

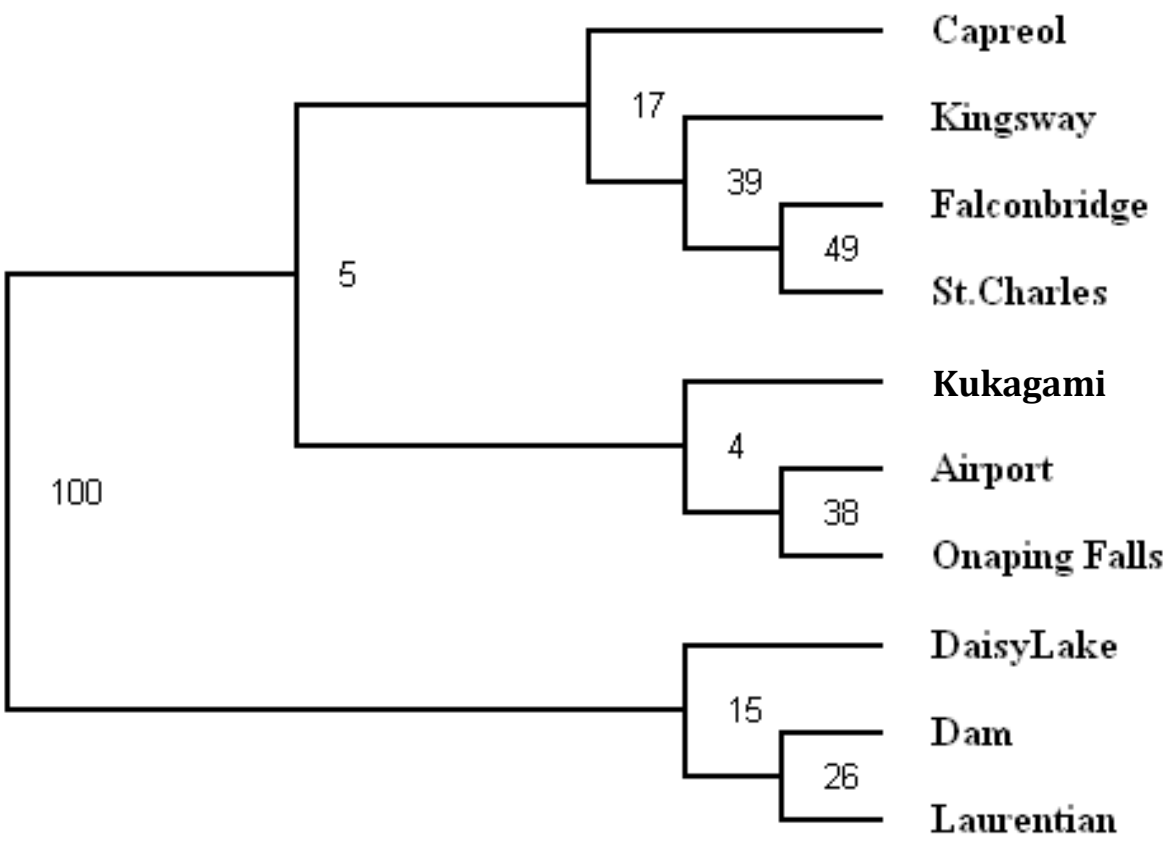

Figure 2.7: Dendogram based on RAPD data for 10 *Q. rubra* populations
(Free Tree Program version 1.50)

Chapter 3: Expression of Genes Associated with Nickel and Copper Resistance in Red Oak *(Quercus rubra)* Populations from a Metal-Contaminated Region in Northern Ontario.

3.1.0 Introduction

Although many studies have reported mechanisms of resistance to metals in herbaceous species, very little is known for hardwood species. These mechanisms vary widely among species, however, there is consistency among taxa for some mechanisms such as: the retardation of absorption of metals, the storage of metals in trichomes of epidermis to avoid the direct effect of metals on the mesophyll, precipitation and chelation of metals in special sites in the plant for detoxification, a rise in the activity of enzymes involved in the removal of free radicals and the production of specific proteins involved in reducing the impact of metals (Cheng 2003).

Genes associated with either Ni or Cu resistance in model and non-model plant species have been identified in many studies. The Ni resistance genes include 1-aminocyclopropane-1-carboxylic acid deaminase (ACC), high affinity nickel transporter family protein (AT2G16800), glutathione reductase (GR), glutathione-s-transferase (GST), iron-regulated protein (IREG), nicotianamine synthase (NAS3), metal transporters (NRAMP), putative transmembrane protein (TMP), serine-acetyltransferase (NAS3), thioredoxin family protein and a zinc transporter of *A. thaliana* (ZAT11) (Table 3.0). Genes involved in Cu resistance include copper transporter protein (COPT1), multi-drug resistance associated protein (MRP4), metallothionein (MT2B) and copper-transporting ATPase (RAN1) (Table 3.1). The specific objective of this study was to investigate the expression of novel genes in *Q. rubra* growing in soil contaminated with different levels of nickel and copper.

3.2.0 Materials and Methods

3.2.1 Sampling

Q. rubra leaf, root and soil samples were collected from six populations around the GSR. Ten trees representing each targeted population were selected for the study. The populations included three contaminated populations: Kelly Lake, Laurentian and Wahnapitae Hydro Dam located within ten kilometers of Sudbury smelters and three reference populations: Capreol, St-Charles and Killarney located as far as 120 kilometers away from Sudbury smelters. Leaf and root samples were wrapped in aluminum foil, immersed in liquid nitrogen and stored at -20°C until DNA and RNA extraction.

3.2.2 Molecular Analysis

DNA and RNA Extraction

Genomic DNA and RNA were extracted from frozen root material using the 2x CTAB extraction protocol as described by Mehes-Smith *et al*. (2007), a modification of Doyle and Doyle (1987) procedure. These modifications included the addition of 1% polyvinyl pyrrolidone (PVP) and 0.2% 2-mercaptoethanol to the cetyltrimethylammonium bromide (CTAB) buffer solution, two additional chloroform centrifugation steps of 10 minutes prior to the isopropanol spin and no addition of RNase. After extraction, DNA was stored in the freezer at -20°C and RNA was stored at -80°C.

RNA samples were tested for quality and intactness by gel electrophoresis on 1% agarose gels. A mixture of 5µL of stock RNA, 2µL of 6x loading buffer and 5µL of TE buffer was loaded into the wells of the agarose gel and was run at 64V for 90 minutes. Gels were then visualized under an ultra-violet light source, documented with Bio-Rad ChemiDoc XRS™ system and analyzed

with Image Lab Software™.

RNA quantitation was performed using the Quibit® RNA BR Assay kit by Life Technologies. The quality of the RNA was then verified on a 1% agarose gel in 0.5x TBE and pre-stained with 1μL of ethidium bromide. The samples of RNA were bulked together to total 10μg per population.

RT-qPCR

RNA was treated with DNase 1 from Life Technologies, then samples were run on a 1% agarose gel to test the effectiveness of the treatment. Reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit from Life Technologies. PCR was performed on both *Q. rubra* DNA and cDNA using the protocol described by Mehes-Smith *et al* (2007). The samples were then run on a 1% agarose gel to analyze the efficiency of the cDNA treatment.

PCR primers previously designed for a study on white birch (*Betula papyrifera)* using the dwarf birch *(Betula nana)* genome were screened on *Q. rubra* DNA and cDNA using PCR. The amplified products were then separated using a 1% agarose gel. Other primers were designed using the previously sequenced cork oak (*Quercus suber*) genome. When possible, primers were designed to span the exon-exon border of the gene and verified to ensure they did not contain hairpins, self-dimers or hetero-dimers. Only primers that showed a reproducible band of the appropriate size were selected for real-time quantitative polymerase chain reaction (RT-qPCR) (Table 3.2-3.5).

RT-qPCR was performed using the Dynamo HS SYBR Green qPCR Kit by Life Technologies and following the manuals protocol. Amplification was conducted using the MJ research PTC-200 Thermal Cycler in triplicates which involved: 1) initial denaturation step at 95°C for 15 minutes 2) denaturation at 94°C for 30 seconds, 3) annealing step for 30 seconds, 4) elongation at 72°C for 30 seconds (repetition of steps 2-4 for 41 cycles 5) melting curve reading at each temperature from 72°C to 95°C for 10 seconds 6) final elongation at 72°C for 3 minutes. This protocol was repeated three separate times for each primer in triplicate for reproducibility purposes.

Data was analyzed using the MJ Opticon Monitor 3.1 by Bio-Rad and delta C (t) values were exported to excel. Delta C (t) values were normalized using a housekeeping gene and the relative expression was calculated.

3.2.3 Statistical Analysis

Data produced by the MJ Opticon Monitor was analyzed using SPSS 20 for Windows and all values were log10 transformed to achieve a normal distribution. A variance-ratio test was conducted with an assumption of data normality. A one-way ANOVA and Tukey's HSD multiple comparison analysis was performed to determine significant differences among means ($p \leq 0.05$).

3.3.0 Results

RT-qPCR

Primers that were used to amplify housekeeping and targeted genes are listed in Table 3.2-3.5. Three of the seven primer pairs targeting housekeeping genes generated strong bands. They include α tubulin 1, cyclophilin 2, and 18rRNA1 (Appendix 4). Primer pairs designed for ZAT11

and GST genes associated with Ni in other species were suitable for RT-q-PCR. When all samples were tested with RT-q-PCR, GST generated multiple bands and was subsequently removed from the study (Appendix 5). The amplification plot resulting from RT-q-PCR from ZAT11 gave a consistent and repeatable band, which can be visualized by the amplification plot in Appendix 6. There was a 120x increase of ZAT11 expression in samples from Wahnapitae Hydro Dam compared to other metal contaminated sites (Laurentian and Kelly Lake). Surprisingly, this gene was also 16x up regulated in samples from uncontaminated site of Capreol compared to the uncontaminated site St. Charles and contaminated sites Kelly Lake and Laurentian (Figure 3.0).

3.4.0 Discussion

A number of different metal resistance mechanisms have been discovered which include the transport-mediated sequestration of metals (Schat and Vooijs 1997). Recently, genes have been found in the *Arabidopsis thaliana* species, which encode proteins with six transmembrane proteins and a long C-terminal cytoplasmic domain. This structure is characteristic of a wide variety of proteins with known function in metals transportation across cellular membranes. For example, the ZRC1 gene from the yeast *Saccharomyces cerevisiae* encodes a protein with the same transmembrane regions and when this gene is overexpressed, the plant is able to resist elevated levels of Zn and Cd (Kamizono *et al*. 1989). Several genes homologous to the ZRC1 have been found including COT1 involved in Co accumulation in yeast (Conklin *et al*. 1992), ZnT-1 involved in transporting Zn out of mammalian cells (Palmiter and Findley 1995) and ZnT-2 which facilitates vesicular sequestration of Zn in mammalian cells (Palmiter *et al*. 1996).

ZAT, which encodes a putative Zn transporter first isolated from *A. thaliana* cDNA is very

closely related to ZnT-1 and ZnT-2. This gene encodes for a protein, which contains 398 amino acid residues and contains the six predicted transmembrane proteins and C-terminal cytoplasmic domain. In *A. thaliana*, ZAT mRNA was overexpressed constitutively throughout the plant and resulted in a significant increase in Zn tolerance, enhanced accumulation of Zn in roots and high external Zn concentrations. This indicated that ZAT function was most closely related to the ZnT-2 gene in mammalian cells (van der Zaal *et al*. 1999).

The family of ZAT includes 20 members spanning from ZAT1 to ZAT20. The function of all ZAT proteins have not been revealed however, it is known that ZAT6 is involved in repression of primary root growth and changes in phosphate acquisition (Devaiah *et al*. 2007), ZAT7 is involved in enhanced tolerance to salt stress (Ciftci-Yilmaz *et al*. 2007), ZAT10 is involved in positive and negative regulation of numerous abiotic stresses (Mittler *et al*. 2006) and ZAT12 is involved in reactive oxygen species and abiotic stress signaling (Rizhsky *et al*. 2004).

ZAT11 is a C_2H_2 zinc finger protein of *A. thaliana* and a nuclear localized transcriptional regulator. ZAT11 positively regulates primary root growth at normal conditions and negatively regulates Ni^{2+} tolerance at excess levels of Ni. Mechanisms of reduced tolerance of Ni^{2+} is believed to involve the repression of transcription of a vacuolar Ni^{2+} transporter gene (Liu *et al*. 2014).

A study conducted by Theriault *et al*. (2016) analyzed the expression of ZAT11 in white birch (*Betula papyrifera*) treated with low doses (5.56mg/kg) and high doses (1600mg/kg) of Ni. ZAT11 expression was 25% and 36% lower in plants treated with a low and high dose of Ni,

respectively when compared to control (untreated). Three groups of genotypes were also observed at the high dose treatment, highly resistant to Ni, moderately susceptible and susceptible. There was an increase of ZAT11 expression by 55% in the resistant plant compared to susceptible and moderately susceptible plants. This suggests that very small doses of Ni are enough to trigger ZAT11 repression and that ZAT11 may also play a role in nickel resistance. This could be through modulation genes involved in the inhibition of oxidative stress.

The present study revealed a 120x up-regulation of ZAT11 in samples from the contaminated population of Wahnapitae Hydro Dam compared to samples from other contaminated populations. The reference population of Capreol also showed a 16x up-regulation of ZAT11 compared to other reference populations. Based on these results, no conclusion could be made on the role of ZAT11 in Ni^{2+} tolerance in the *Q. rubra* species. The lack of amplification with other primer pairs might be due to absence of primer binding sites or weak primer binding due to low complementarity to *Q. rubra* cDNA.

Further analysis will be required to determine the significance of the ZAT11 up-regulation in the Wahnapitae Hydro Dam and Capreol populations. A gene expression study on *Q. rubra* leaves collected during the sampling process in this study would be beneficial to verify reproducibility of results. To determine the exact role of ZAT11 in nickel resistance in *Q. rubra*, a controlled experiment in growth chambers similar to the study conducted on the expression of ZAT11 in white birch (*Betula papyrifera*) would be required (Theriault *et al*. 2016a).

Table 3.0: Candidate genes involved in Ni resistance in model and non-model plant species

Gene	Species	Reference
ACC	*Brassica napus*	Stearns *et al*., 2005
AT2G16800	*Arabidopsis thaliana*	Stearns *et al*., 2005
IREG	*Arabidopsis thaliana*	Schaaf *et al*., 2006
GR	*Thlapsi goeisngense*	Freeman *et al*., 2004
GST	*Thlapsi goesingense*	Freeman *et al*., 2004
	Betula papyrifera	Theriault *et al*., 2016
NRAMP 1-4	*Thlaspi japonicum*	Mizuno *et al*., 2005
	Noccaea Caerulescens	Visioli *et al*., 2012
	Thlaspi caerulescens	Wei *et al*., 2008
	Betula papyrifera	Theriault *et al*., 2016
NAS3	*Noccaea Caerulescens*	Visioli *et al*., 2012
	Thlapsi goesingense	Mari *et al*., 2006
TMP	*Betula papyrifera*	Theriault *et al*., 2016
SAT	*Thlapsi goesingense*	Freeman *et al*., 2004
Thioredoxin	*Chlamydomonas reinhardtii*	Lemaire *et al*., 2004
	Betula papyrifera	Theriault *et al*., 2016
ZAT11	*Arabidopsis thaliana*	van der Zaal *et al*., 1999

Table 3.1: Candidate genes involved in Cu resistance in model and non-model plant species

Gene	Species	Reference

RAN1	*Arabidopsis thaliana*	Kobayashi *et al*., 2008
MRP4	*Betula pendula*	Keinänen *et al*., 2007
COPT 1	*Arabidopsis thaliana*	Sancenon *et al*., 2004
MT2B	*Arabidopsis thaliana*	Guo *et al*., 2008

Table 3.2: Sequences of *Q. rubra* primers, designed from Ni resistant gene, using the Dwarf birch (*Betula nana*) genome, screened for RT-qPCR

Target	Melting temp (° C)	Primer	Expected amplicon (bp)	PCR product in DNA (bp)	PCR product in cDNA (bp)
ACC	F: 66.96	F: TGGCAATCTCCTGGTTGAGCGT	315	Multiple	
	R: 66.44	R: GGGATCATGGCTGAGTACATTGAAGG			
AT2G16800	F: 64.68	F: GAGCTCTCTGGGGGTGTGGC	335	335	Multiple
	R: 64.65	R: TGCCGGCACGACCATCATCA			
GR	F: 64.57	F: AGCGGTTATTGACGAATTCTGGGGT	169	None	
	R: 65.46	R: TGGCCAGGGATAGGGGGACG			
GST	F: 60.0	F: TCTGAAACTCAAGGGTGTTGATT	103	None	
	R: 60.0	R: GACTGGAACCTTTTTGTGAACTG			
IREG1	F: 64.82	F: CAGAAGGCCATCCTCCAGAGAAGC	149	Multiple	
	R: 64.36	R: CCAGAGTGCCAAAGCCACAGC			
NAS3	F: 65.03	F: AGGCTCTGTGGGGAGGCAGA	320	500	Multiple
	R: 64.94	R: GAGAAAAGCCCGAGCCCCGT			
NRAMP 1	F: 60.0	F: TACATTCTCGCCGTCATTTATCT	215	None	
	R: 60.0	R: GTTGATGCCTTTGATCTTGAAAC			
NRAMP2	F: 60.0	F: CTAGCAAGATCAGAGAGATGGGA	201	None	
	R: 60.0	R: GAAACTTTCTCCATCCTGGTTTC			
NRAMP3	F: 62.90	F: GTTTTGCCTCTCTGGGTGG	307	None	
	R: 62.70	R: GTTGGGAGCAATCTTTCTTGACTGT			
NRAMP4	F: 71.07	F: TCCTCTCAGCCAGGGTCGGGGT	350	None	
	R: 71.49	R: AAGTTCCACGCCATTGGGCTTCGTT			
SAT	F: 66.01	F: GTGGATATCCATCCGGGAGCTAAGA	326	Multiple	
	R: 67.34	R: GACGGCCGCTCCTTCCCTC			
Thioredoxin	F: 60.0	F: GAAAAGCTTCTTCAGATCTGGGT	221	None	
	R: 60.5	R: GACTTGGCCTTTCTAAAACTTGC			
TMP	F: 60.0	F: TTCTAATAAGGTATTGTGCGCGT	114	None	
	R: 60.0	R: GGAGGAAAAGATTCACCAAGAGT			
Ton B family protein	F: 61.6	F: AGGTTCTGAAGCAGGCTCGTAT	139	None	
	R: 59.6	R: AGAACCAGAACCAGAACCAGAC			
Ton B receptor	F: 60.0	F: TCATGAACCTCGATGTCATACTG	293	None	
	R: 60.0	R: GCATATCCCAAGATCTACAGTGC			
ZAT11	F: 64.61	F: ACCGAGCCAGCCACAAGAG	149	149	149
	R: 65.69	R: CCGCCCAAAGCTTGCCCCA			

Table 3.3: Sequences of *Q. rubra* primers, designed from Cu resistant genes, using the dwarf birch (*Betula nana)* genome, screened for RT-qPCR

Target	Melting temp (°C)	Primer	Expected amplicon (bp)	PCR product in DNA (bp)	PCR product in cDNA (bp)
COPT1	F: 68.34	F: GCACATGACCTTCTTCTGGGGCA	303	303	Multiple
	R: 69.30	R: AACCCAACGGCGTGGCCAG			
MRP4	F: 63.46	F: GCTTGATCCTCTGCCTTTCTACTTG	380	None	
	R: 63.67	R: CCACTTCCTGTTCGACCAACAAC			
MT2B	F: 65.83	F: CTTGTGGAGTTCAAAGGCGGAAAG	387	None	
	R: 65.02	R: GGCAGCCAAGCTGACAGTATGAAC			
RAN1	F: 63.73	F: CCTTGTGCTTTGGGTCTGGC	337	Multiple	
	R: 62.51	R: GCTATTGTTATCGGCATCCTTGG			

Table 3.4: Sequences of *Q. rubra* primers, designed from Ni resistant genes in the cork oak (*Quercus suber*) genome screened for RT-qPCR

Target	Melting temp (° C)	Primer	Expected amplicon (bp)	PCR product in DNA (bp)	PCR product in cDNA (bp)
GST	F: 60.0	F: ACTGCTGCCTCAACATCCTT	276	276	276
	R: 60.0	R: GAAGTTGGGTCCATGCAGAT			
NRAMP1	F: 60.0	F: CACAAAGATGGGACCACACA	113	None	
	R: 60.0	R: GAGCACAGGCTTTGTGGATT			
Thioredoxin	F: 60.0	F: CGAGAAAGAGGTCGGTCAAG	136	None	
	R: 60.0	R: CCAAGTCTGTTTTGATCGCA			

Table 3.5: Sequences of *Q. rubra* primers, designed from housekeeping genes, screened for RT-qPCR

Target	Melting temp (° C)	Primer	Expected amplicon (bp)	PCR product in DNA (bp)	PCR product in cDNA (bp)
18rRNA 1	F: 67.70 R: 66.86	F: ATGCCGGCGACGCATCATT R: CACTACCTCCCCATGTCAAGATTGGA	186	186	186
18rRNA 2	F: 64.77 R: 65.50	F: GTGGTGACAAGTGACGGAGAATTAGG R: GCCGGTAGAAGGGACGAGCA	350	350 (weak)	350
α-tubulin 1	F: 65.10 R: 65.16	F: TGTTGACTGGTGCCCACCG R: CACAAAGGCGCGCTTGGCAT	187	187	187
α-tubulin 2	F: 71.37 R: 69.98	F: TGCCTCGAGCACGGCATCCA R: GATCCGAAACCGGAACCTGTTCCAC	378	Multiple bands	
Cyclophilin 1	F: 69.52 R: 69.75	F: TGGGCGGATCGTGATGGAGC R: TCCCGGCAGTGAAGTCGCCTC	183	Multiple bands	
Cyclophilin 2	F: 64.78 R: 65.10	F: TGGGCGGATCGTGATGGAGC R: CACGACCTGGCCGAACACCA	370	200	200
Ef1 α- 1	F: 72.42 R: 72.62	F: GATCTCGGAGCCCAAGAGGCCCAC R: GGCAATCCAAGACGGGCGCG	378	Multiple bands	

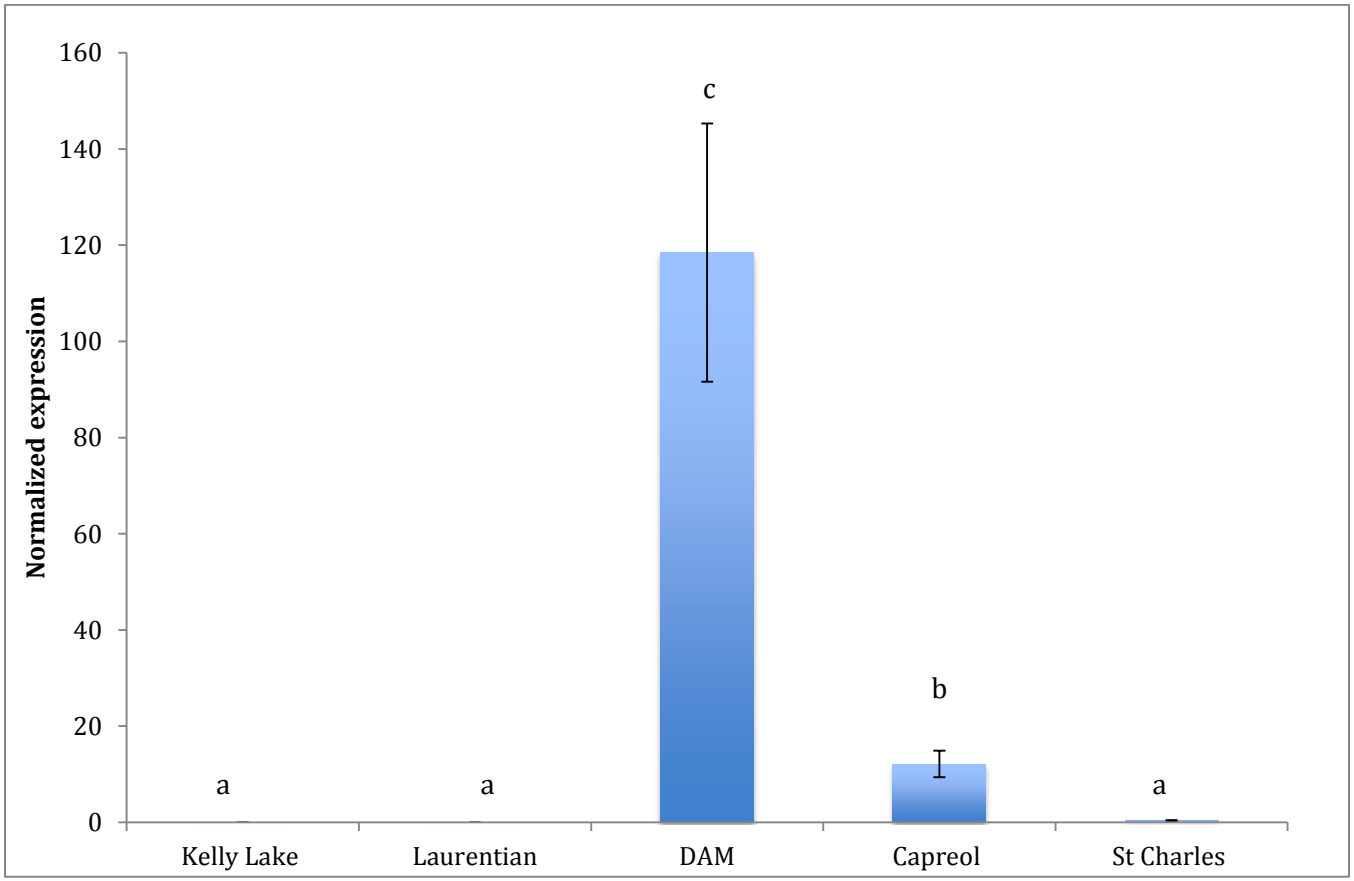

Figure 3.0: Expression of ZAT11 in Q. rubra in contaminated (Kelly Lake, Laurentian Wahnapitae Hydro Dam) and reference (Capreol, St-Charles) populations of the Greater Sudbury Region. Expression of ZAT11 was standardized based on the housekeeping gene α-tubulin. Significant differences were found among groups using the Tukey's multiple comparisons t-test ($p < 0.05$)

Chapter 4: General Conclusions

Metal toxicity is a major cause of abiotic stress in plants. It has been established that changes in genetic variation occur very rapidly in plants in response to environmental stressors such as increased levels of metals. *Q. rubra* is one of the pioneer species that survived the high level of metal contamination in mining regions in Northern Ontario (Canada). The main objectives of the present study were to 1) determine the level of genetic variation in *Q. rubra* populations from mining damaged ecosystems using a RAPD marker system and 2) analyze the level of gene expression of candidate genes for nickel and copper resistance.

Genetic diversity parameters revealed high levels of diversity within populations suggesting a predominance of genetic recombination within each population and a great indication of population sustainability. Low values of inter-population differentiation and high gene flow suggest limited chances of population divergence overtime. The estimated high gene flow is likely a combination of pollen dispersal and seed distribution through animal dispersers. No significant difference in genetic variation was found among the contaminated and reference sites for all the genetic parameters estimated.

A zinc transporter gene of *Arabidopsis thaliana* (ZAT11) involved in negatively regulating Ni^{2+} tolerance and positively regulating primary root growth was differentially expressed in samples analyzed. There was a 120x increase of ZAT11 expression in samples from the metal contaminated population of Wahnapitae Dam compared to other metal contaminated and uncontaminated sites. No association between soil metal levels and expression of ZAT11 was

established and for this reason, further analysis will be required to determine the cause of differential expression.

To determine the exact role of ZAT11, and other Ni and Cu resistant genes in *Q. rubra*, future studies will involve a gene expression using a controlled experiment with different doses of Ni and Cu administered to *Q. rubra* seedlings. Subsequent studies will also include a comparative transcriptome analysis to identify novel candidate genes associated with Ni and Cu resistance.

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Appendices

Appendix 1: Northern Red Oak (*Quercus rubra*)

Appendix 2: Metal concentration guidelines for soil according to the Ontario Ministry of Environment and Energy (OMEE)

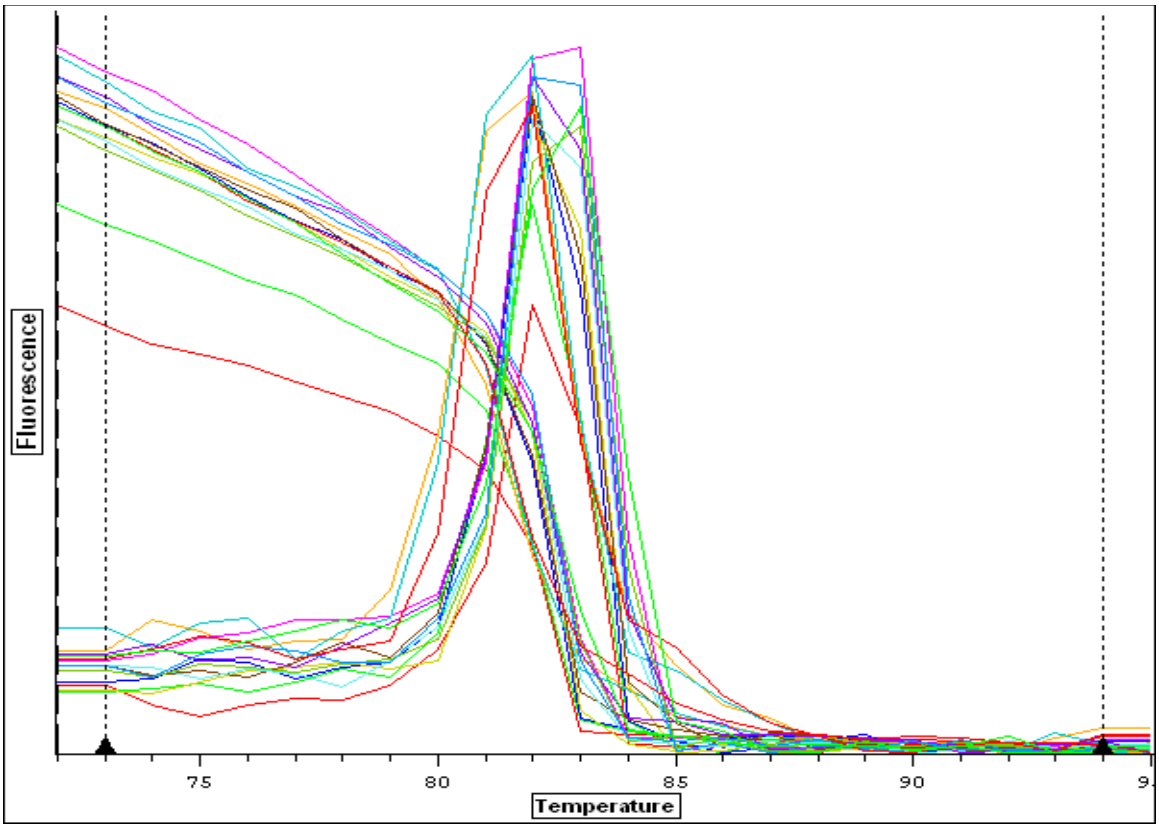
Metals	**Ontario Sediment Quality Guidelines (mg/kg)**	
	Lowest Effect Level	**Severe Effect Level**
Arsenic	6	33
Cadmium	0.6	10
Cobalt		50
Copper	16	110
Iron	2%	4%
Lead	31	250
Manganese	460	1100
Nickel	16	75
Zinc	120	820

Appendix 3: Nucleotide sequence of RAPD primers screened on red oak *(Quercus rubra)* DNA samples

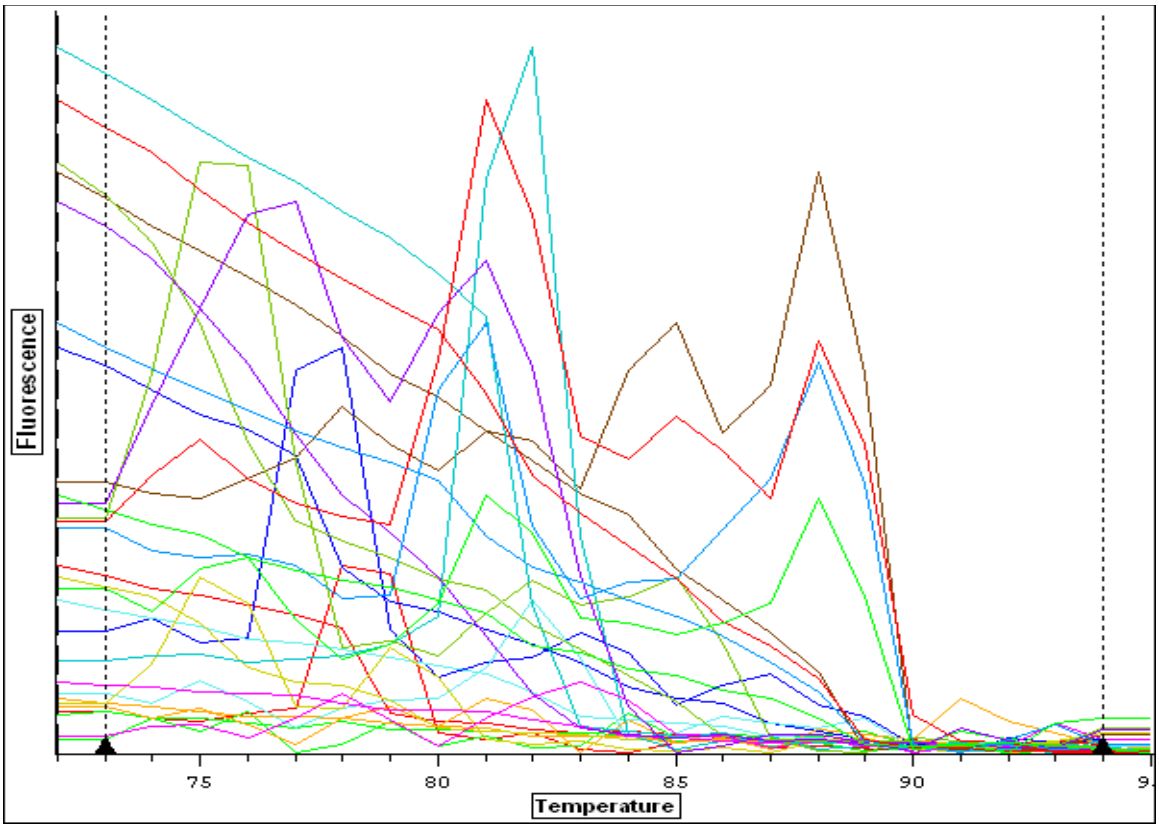
RAPD Primers	5' → 3'	GC content (%)	Amplification Product
E18	GGACTGCAGA	60	Weak
Grasse 1	CCGCCCAAAC	70	None
Grasse 2	GTGGTCCGCA	70	None
Grasse 3	GTGGCCGCGC	90	None
Grasse 4	GAGGCGCTGC	80	Weak
Grasse 5	CGCCCCCAGT	80	Weak
Grasse 7	CACGCCGAGT	70	Weak
Grasse 8	GGGTAACGCC	70	None
Grasse 9	GTGATCGCAG	60	Weak
Grasse 10	CAGCACCCAC	70	None
OPA 1	CAGGCCCTTC	70	None
OPA 2	TGCCGAGCTG	70	Weak
OPA 3	AGTCAGCCAC	60	None
OPA 4	AATCGGGCTG	60	Weak
OPA 5	AGGGGTCTTG	60	Moderate
OPA 6	GGTCCCTGAC	70	None
OPA 7	CAAACGGGTG	60	None
OPA 8	GTGACGTAGG	60	None
OPA 11	CAATCGCCGT	60	None
OPA 12	TCGGCGATAG	60	None
OPA 14	TCTGTGCTGG	60	None
OPA 15	TTCCGAACCC	60	None
OPA 16	AGCCAGCGAA	60	Good
OPA 17	GACCGCTTGT	60	None
OPA 18	AGGTGACCGT	60	Moderate
OPA 19	CAAACGTCGG	60	Good
OPA 20	GTTGCGATCC	60	Weak
OPB 05	AGGGGTCTTG	60	Good
OPB 7	GGTGACGCAG	70	None
OPC 10	TGTCTGGGTG	60	None
OPD 14	CTTCCCCAAG	60	None
OPF 17	AACCCGGGAA	60	None
OPG 12	CAGCTCACGA	60	Weak
OPH 20	GGGAGACATC	60	None

Appendix 3 (continued): Nucleotide sequence of RAPD primers screened on red oak *(Quercus rubra)* DNA samples

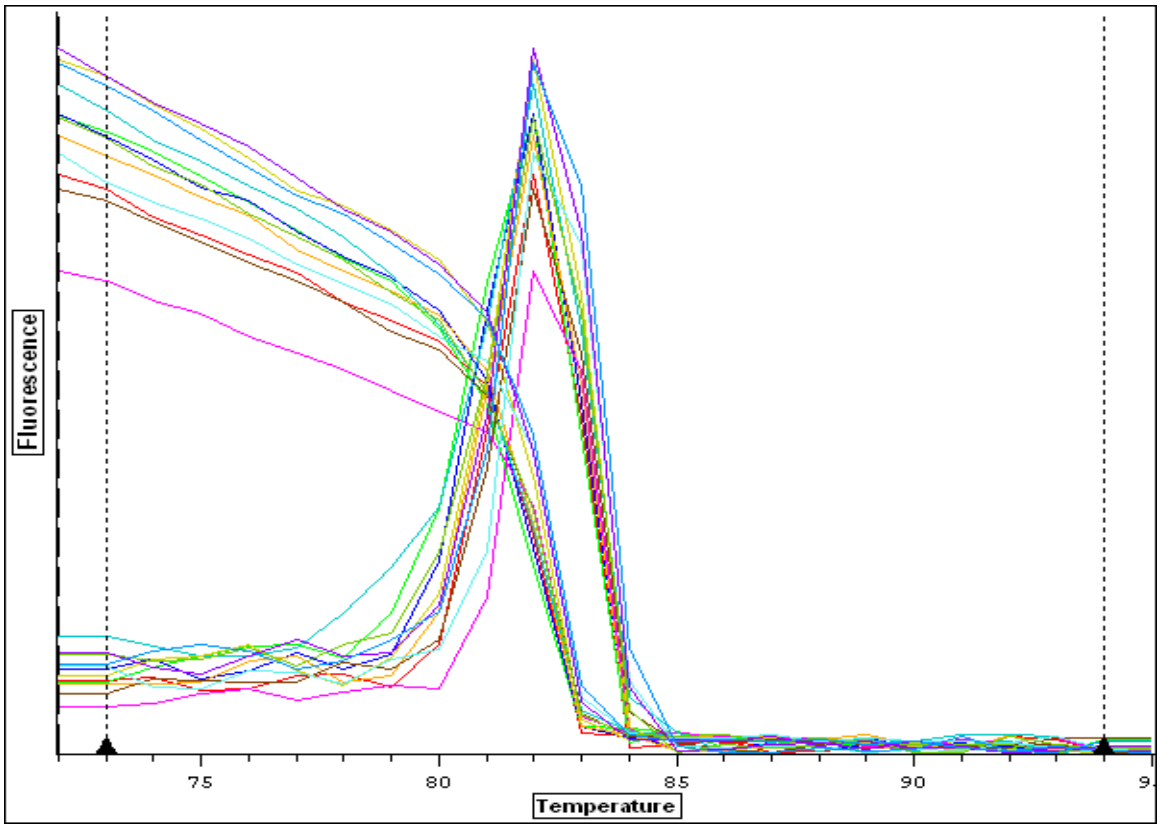
RAPD Primers	5' → 3'	GC content (%)	Amplification Product
OPT 17	CCAACGTCGT	60	Good
OPX 4	CCGCTACCGA	70	None
Primer 9	GTGCGTCCTC	70	None
Pinus 23	CCCGCCTTCC	80	Good
Pinus 146	ATGTGTTGCG	50	Moderate
Pinus 184	CAAACGGCAC	60	Weak
UBC 48	TTAACGGGGA	50	None
UBC 78	GAGCACTAGC	60	None
UBC 184	CAAACGGCAC	60	Weak
UBC 186	GTGCGTCGCT	70	Good
UBC 197	TCCCCGTTCC	70	None
UBC 201	CTGGGGATTT	50	None
UBC 214	CATGTGCTTG	50	Weak
UBC 260	TCTCAGCTAC	50	None
UBC 337	TCCCGAACCG	70	Weak
UBC 372	CCCACTGACG	70	Weak
UBC 402	CCCGCCGTTG	80	Good
UBC 486	CCAGCATCAG	60	None
UBC 494	TGATGCTGTC	50	None
UBC 537	CGAAAGGACT	50	None
UBC 551	GGAAGTCCAC	60	None
UBC 561	CATAACGACC	50	None



Appendix 4: Real-Time PCR amplification plot of housekeeping gene α-tubulin displaying fluorescence as a function of melting temperature.



Appendix 5: Real-Time PCR amplification plot of GST displaying fluorescence as a function of melting temperature.



Appendix 6: Real-Time PCR amplification plot of ZAT11 displaying fluorescence as a function of melting temperature.