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Identification of Nonnative *Phragmites* at Clear Creek Wildlife Management Area, Kentucky, Using Genetic Techniques

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Phragmites australis (Cav.) Trin. ex Steud. (common reed), a perennial wetland grass, has been present in the United States along both Atlantic and Pacific coasts for hundreds of years (Orson 1999, Goman and Wells 2000). *Phragmites* was deemed rare in the 1800s (Saltonstall 2002) and according to Ward (2010), its distribution was discontinuous in portions of the Midwest, southern Canada, and California, and sporadic along the Gulf Coast from Florida to Texas throughout the 1800s. Also, during that time it was considered absent from Virginia to Georgia inclusive, and inland portions of Alabama and Mississippi (Ward 2010).

The abundance and distribution of *Phragmites* has dramatically increased over the last century (Ward 2010) with the most significant increases along the Atlantic coast (Saltonstall 2002). The rapid expansion was primarily due to the introduction of a European strain in the late 1700s or early 1800s via one or more coastal ports along the Atlantic Coast (Saltonstall 2002). It is highly competitive, aggressive, and virtually indistinguishable morphologically from the native strains, resulting in "cryptic" invasions (Carlton 1996, Saltonstall 2002) going undetected for decades in various locations around the country. The European strain's spread was likely facilitated by construction of railroads, major roadways (Saltonstall 2002), and changes to hydrologic regimes and/or nutrient availability (Saltonstall 2002, King et al. 2007). Currently, it dominates and effectively displaces native *Phragmites* in the Northeast (Ward 2010) and has expanded further westward into parts of the Midwest (Saltonstall 2003) and the Great Lakes region (Saltonstall 2002, T'ulbure et al. 2007, Howard et al. 2008, Jodoin et al. 2008, and Whyte et al. 2008).

Currently, *Phragmites* is sporadically distributed throughout Kentucky, but mostly occurs in western counties (Liang 2010, USDA 2013). Historically, *Phragmites* occurred in western counties (Mitsch et al. 1983a) and based on herbarium specimens, it was first recorded in Kentucky in Calloway County in 1973 (Liang 2010). Moreover, McFarland (1942) did not include *Phragmites* in the Kentucky catalogue of vascular plants, indicating that *Phragmites* was likely introduced after this survey was conducted. In the Clear Creek Wildlife Management Area (CCWMA) located in Hopkins County in western Kentucky, *Phragmites* is prevalent (Figure 1). Clear Creek is a fifth order stream that is approximately 50 km long that runs westward to feed into the Tradewater River and is considered the largest stream in the area (Mitsch et al. 1983b). A few decades ago, Mitsch et al. (1983b) described numerous wetland vegetation types occurring in the stream channel, shrub-scrub communities in the open sloughs along the channel, and several types of trees composing the surrounding bottomland forests adjacent to the stream. *Phragmites* was not mentioned in the wetland's description, indicating that its occurrence was either at low levels or not detected. Currently, the dominant plant in the wetland is *Phragmites*, particularly in the lower reaches where it grows in dense continuous stands (Figure 1).

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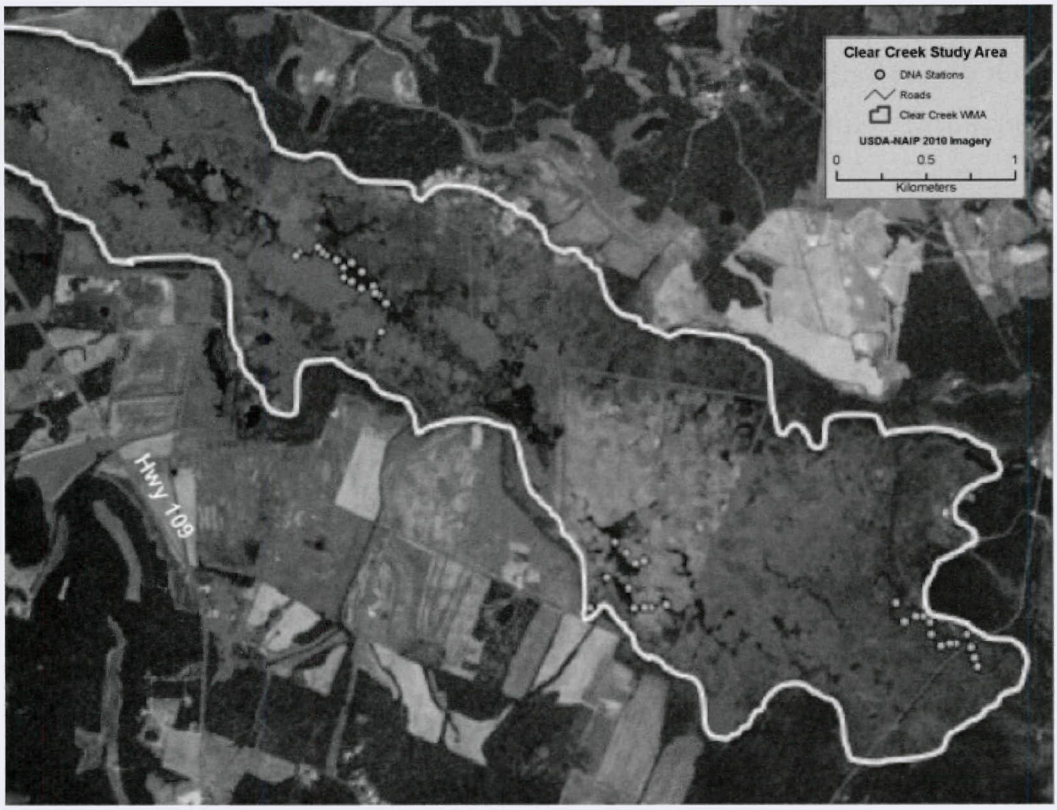

Figure 1. Clear Creek Study Area. Circles indicate sample locations of *Phragmites* plants that were used in DNA analysis. White outline shows extent of *Phragmites* growth within the creek marshland. Highway 109 is indicated.

There are sparse native wetland plants in this area such as duckweed (*Lemna minor* L.), yellow water lily (*Nuphar advena* Ait.), coontail (*Ceratophyllum demersum* L.), arrow arum (*Peltandra virginica* Schott), pond weed (*Potamogeton* sp.), and common cattail (Krzton-Presson 2011).

Clear Creek has been heavily impacted by strip mine activity in the last few decades, which has likely facilitated introduction and spread of *Phragmites*. However, it is unclear whether a nonnative exotic strain or a native strain is present. Given the potential negative ecological impacts expected with continual increase (e.g., loss of native food sources for local fauna; Ailstock et al. 2001) and changes to nutrient storage and cycling (Armstrong and Armstrong 1988, Findlay et al. 2002), management of *Phragmites* at the CCWMA has become necessary. Since differences exist in invasiveness/competitiveness between *Phragmites* strains, an important first step in managing it is to identify which strain has invaded the area.

We took samples of *Phragmites* along Clear Creek from 50 haphazard locations, the majority of which were within the CCWMA. Since boat ramps and access points to the creek were limited, and the *Phragmites* restricted boat travel, samples were taken along the interior edge of Clear Creek using small kayaks (Figure 1). Several new leaves were cut from each plant using a clean razor blade and placed in a plastic ziplock bag with silica desiccant. Each sample was given an identification number, and GPS coordinates were taken to identify sample locations (Figure 1). Leaves were transferred to coin-sized envelopes at the lab, placed in a plastic container that contained silica desiccant, and stored in a refrigerator at 4°C.

We used restriction fragment length polymorphisms (RFLPs) to identify the strain(s) of *Phragmites* at the CCWMA (Saltonstall 2003). DNA was extracted from dried leaves using the DNeasy Plant Mini Kit (Qiagen, Valencia, California) and polymerase chain reaction (PCR) was carried out to amplify two noncoding chloroplast regions of DNA using the primer pairs *trnL* (UAA)5′ "b" and *trnL*bR (Taberlet et al. 1991, Saltonstall 2002), and *rbcL* (Saltonstall 2001) and *rbcL*3R (Saltonstall 2003). PCR reactions were carried out in 50 µl volumes using GoTaq Master Mix (Promega, Madison, Wisconsin), with final concentrations of 1X Master Mix, 0.1µM of each primer, and <250 ng of DNA. Ten µl of PCR product was digested with either *Rsa*I (*trnL*b regions) or *Hha*I (*rbcL* region), according to the manufacturer's protocol (Madison). Restriction fragments were visualized on 3% TAE agarose gels stained with ethidium bromide.

Of the 50 *Phragmites* samples collected from Clear Creek, 48 had successful DNA extraction. Based on successful and consistent PCR amplification and restriction fragment analysis, 45 samples were used for *Phragmites* strain identification. In all 45 plants, *Hha*I cut the 350 bp *rbcL* region at 104 bp and *Rsa*I left the 350 bp of the *trnL*b region intact (Figures 2A and B). The restriction fragment analysis indicated that all analyzed plants have an invasive haplotype and are of nonnative origin.

An herbarium study conducted by Liang (2010) demonstrated that *Phragmites* has quickly spread throughout western Kentucky in the last few decades; however, the type of *Phragmites* was not indicated. Invasive *Phragmites* has been documented in other parts of the Midwest (Saltonstall 2002) and our study shows unequivocally that invasive *Phragmites* exists in the CCWMA and thus, western Kentucky. Saltonstall's (2003) method for identifying the different strains was reliable, reproducible, low-cost, and the methodology was easily followed, but surprisingly few published studies have applied this technique to wetland management questions (e.g., Saltonstall 2003, T'ulbure et al. 2007). To direct the course of management in wetland systems, we advocate the use of Saltonstall's (2003) technique to assist in differentiating among *Phragmites* types.

Multiple lines of evidence indicate that invasive *Phragmites* can spread quickly (see Bart and Hartman 2003; League et al. 2006; King et al. 2007; Howard et al. 2008; Kettenring et al. 2010, 2011; Kulmatiski et al. 2011). Since *Phragmites* is already well established at the CCWMA, the primary concern is to control patch size. In large patches, high levels of genetic diversity will likely accumulate due to increased sexual reproduction (Kettenring and Whigham 2009, McCormick et al. 2010, Kettenring and Mock 2012), which in turn increases the amount of seeds available resulting in a positive feedback system involving nutrient levels, patch size and genetic diversity (Kettenring et al. 2010, 2011) leading to rapid spread (McCormick et al. 2010). Large stands of *Phragmites* occur at the CCWMA, facilitating high levels of genetic diversity and seed production. Additionally, Kettenring and Whigham (2009) and Kettenring et al. (2011) suggest controlling/eradicating small, satellite patches of invasive *Phragmites* because as they grow, large sufficient genetic diversity will accumulate for viable seed production, thus serving as a point for future invasions (McCormick et al. 2010). In addition to controlling patch size and development, management practices that prevent seed production and decrease nitrogen levels may curb progression of invasive *Phragmites* (King et al. 2007, Kettenring et al. 2010, Kettenring et al. 2011) at the CCWMA. For example, mowing plants in midseason followed by fall herbicide treatment would prevent seed production (Kettenring et al. 2010) and coordination between managers of the watershed and those parties that contribute nutrients to the watershed could lessen nitrogen levels (Kettenring et al. 2011), thus restricting the positive feedback loop that may result.

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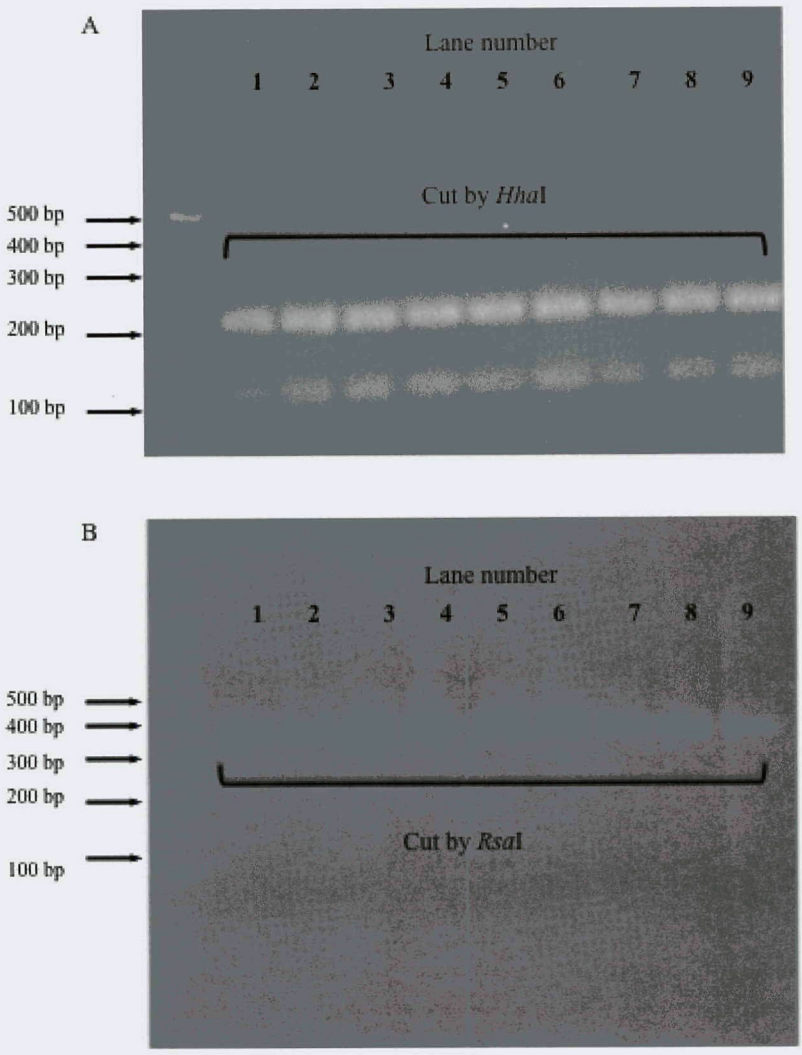

Figure 2. Restriction fragment length polymorphism (RFLP) band patterns of nine *Phragmites* samples collected at Clear Creek Wildlife Management Area (CCWMA) obtained using Saltonstall's (2003) method which differentiates between strains based on where the restriction enzymes cut. In inland native plants, *Rsa*I will cut at 282 bp of the 350 bp *trnL*b region, but will not cut nonnative plants or the Gulf Coast strain. In nonnative plants *Hha*I will cut at 104 bp of the approximate 350 bp *rbcL* region, but will not cut either inland native plants or Gulf Coast plants. Lane 1 contains a 100 bp ladder (Promega). All other lanes contain nonnative strains as evidenced by the cuts made by restriction enzymes. (A) *rbcL* region cut with *Hha*I; (B) *trnL*b region cut with *Rsa*I.

aerial photographs of the Clear Creek area. We also thank Tyler Smith and two anonymous reviewers for helpful comments in revising this note.

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