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MOLECULAR AND DENDROCHRONOLOGICAL ANALYSIS OF NATURAL ROOT GRAFTING IN *POPULUS TREMULOIDES* (SALICACEAE)[1]

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Trembling aspen (*Populus tremuloides*) is a clonal tree species, which regenerates mostly through root suckering. In spite of vegetative propagation, aspen maintains high levels of clonal diversity. We hypothesized that the maintenance of clonal diversity in this species can be facilitated by integrating different clones through natural root grafts into aspen's communal root system. To verify this hypothesis, we analyzed root systems of three pure aspen stands where clones had been delineated with the help of molecular markers. Grafting between roots was frequent regardless of their genotypes. Root system excavations revealed that many roots were still living below trees that had been dead for several years. Some of these roots had no root connections other than grafts to living ramets of different clones. The uncovered root systems did not include any unique genotypes that would not occur among stems. Nevertheless, acquiring roots of dead trees helps to maintain extensive root systems, which increases the chances of clone survival. Substantial interconnectivity within clones as well as between clones via interclonal grafts results in formation of large genetically diverse physiological units. Such a clonal structure can significantly affect interpretations of diverse ecophysiological processes in forests of trembling aspen.

Key words: aspen; clonal integration; dendrochronology; diversity; microsatellites; natural root grafting; *Populus tremuloides*; root system; Salicaceae.

Numerous tree species combine sexual and vegetative propagation, depending on the variation in the limiting biotic and abiotic factors in their distribution ranges (Eckert, 2002). In trembling aspen (*Populus tremuloides* Michx.), the balance between the two modes of reproduction is clearly shifted toward vegetative propagation through root suckering after a disturbance (Barnes, 1966; Perala, 1990). Theory has predicted that limited recruitment of seedlings in clonal species leads to a decline of their clonal diversity over time (Shapcott, 1995; Pornon et al., 2000; Moriguchi et al., 2001; Balloux et al., 2003). Nevertheless, recent studies addressing the effect of vegetative propagation on population structure failed to find such a decline (Mayes et al., 1998; Erickson and Hamrick, 2003; Pluess and Stocklin, 2004). Our findings from the southern boreal forest of eastern Canada indicated that aspen maintains a high clonal diversity along the successional gradient despite its vegetative propagation mode (Namroud et al., 2005, 2006).

These results bring about a question of how clonal diversity is maintained in species with extensive clonality over long periods. Montalvo et al. (1997) proposed that high diversity values could be explained by somatic mutation accumulation. Other authors asserted that high clonal diversity can be maintained by episodes of sexual reproduction no matter how rare they are (Jelinski and Cheliak, 1992; Persson and Gustavsson, 2001; Kjølner et al., 2004). An additional mechanism that may contribute to the clonal diversity maintenance could lie in the structure and development of the root system.

Through clonal growth, aspen's root system integrates roots of two types: old parental roots that produced suckers and new roots grown from suckers (DeByle, 1964; Strong and LaRoi, 1983; Desrochers and Lieffers, 2001). Part of the original parental root system continues to live even in mature aspen stands and connects ramets that have regenerated from the same parental roots (Desrochers and Lieffers, 2001). However, the level of ramet integration into a communal root system may increase even further by natural root grafting (Barnes, 1966; Shepperd, 1993; Desrochers and Lieffers, 2001). Natural root grafting is a rarely studied but frequent phenomenon that results in a morphological union of cambium, phloem, and xylem of two or more previously distinct roots (Graham and Bormann, 1966). Such a union may occur between roots of the same or different genotypes, forming intra- or interclonal grafts. Nevertheless, clonal identity of roots involved in graft formation in trembling aspen has not yet been verified.

Moreover, grafts have been observed not only between roots of two living trees but also between living trees and dead stumps or snags (Graham and Bormann, 1966; Eis, 1972; Stone, 1974; Desrochers and Lieffers, 2001; Fraser et al., 2007). Survival of such living roots on dead stumps (LRDS) can exceed several decades and presumably depends on an influx of assimilates and other substances from the living stems to which they have grafted (Greenidge, 1955; Eis, 1972; Desrochers and Lieffers, 2001; Fraser et al., 2006).

In the current study, we hypothesized that physiological integration of different clones through root grafts may allow these clones (genotypes) to remain alive after the death of the corresponding stems. Potentially, a disturbance removing the aboveground part of the stand could restimulate suckering in these root segments, thereby allowing these genotypes to persist after they would normally have been eliminated. From this perspective, aspen's communal root system could constitute a "genotype bank." To validate this hypothesis, three aspen stands were

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excavated and the uncovered root systems were examined for the presence of (1) root grafts, in particular the interclonal grafts; (2) living roots on dead stumps; and (3) unique genotypes having no corresponding ramets among the living stems. Dendrochronological and molecular analyses, two different approaches rarely combined in the past, were employed in this study to reconstruct the root system development with respect to grafting and to delineate the clones.

Findings of the current study may be interesting not only for the researchers investigating the processes of diversity maintenance in clonal plant species but also for a large community of scientists exploring diverse ecophysiological processes in natural aspen stands. We identified many factors influencing these dynamic processes under the assumption that stands were composed of discrete individuals or genetically uniform clones. Our results indicated that some of these conclusions may be confounded by the physiological integration of different clones into a communal root system.

MATERIALS AND METHODS

Study area—The three study sites (M, W, and K) were located in northwestern Quebec, Canada, in the boreal forest at latitudes between 48°11′N and 48°30′N and longitudes between 78°45′W and 79°23′W. This area is part of the Northern Clay Belt characterized by post-Wisconsinian lacustrine deposits forming heavy clay soils. Climate of the region is cold and continental with an average annual temperature of 0.8°C, mean yearly precipitations of 857 mm, and a mean annual frost-free period of 64 d (Environment Canada, 1993).

Site parameters had to allow for root excavation; thus, the sites had to have a gentle slope and a nearby source of water (DesRochers and Lieffers, 2001). To excavate the root systems of the maximum number of ramets within minimum-size areas without intermingling of roots of other tree species, we selected early successional, even-aged, pure aspen stands. Moreover, because the incidence of root grafts is expected to increase with stand age (Stout, 1961; Basnet et al., 1993) while aspen stem density decreases with age (Brassard et al., 2008), 20–40-year-old stands were chosen. The three stands meeting these requirements had regenerated from root suckers after clear cutting, had a minimum density of 0.5 trees per square meter, and lacked coniferous undergrowth. A plot of ca. 30 m^2 was established in each of the three sites. Plots were placed in such a way that they encompassed the maximum number of clones that were identified with preliminary microsatellite analysis.

Excavation and sampling—In August 2006, all stems within the plots were cut down, and the root system was uncovered with a jet of water generated with a forest fire pump. The depth of the mineral horizon into which plots were excavated varied between 30 and 60 cm with site M being the deepest one. This technique enabled fast removal of large volumes of soil so most of the root system could be exposed and accurately mapped.

Bark samples from all the stems, LRDS, and from both sides of grafts were taken and stored at −80°C until DNA extraction. To be able to age the grafts and distinguish the original parental roots (all roots older than the corresponding ramet) and roots grown from suckers (roots the same age as the corresponding ramet or younger), we removed cross-sectional disks at breast height and at ground level from all the stems and from every root that was at least 2 cm in diameter at its point of insertion into the stump.

Grafts were collected, dried, and cut to verify whether the grafted roots were in a real physical union by sharing common growth rings. Moreover, by dating the growth of the first common ring, the age of roots when grafting was completed could be determined.

Dendrochronological analysis—Grafted roots were cut into cross sections throughout the length of the grafts. Stem, root, and graft cross sections were then dried and sanded with a progressively finer grit paper starting with grade 150 and finishing with 500. To further improve visibility of growth rings, we cut the disk surface with a razor blade, and the wood vessels were filled up with white chalk. Ring widths were measured with a Velmex (Bloomfield, Indiana, USA) micrometer with a precision of 1 μm. Because root radial growth is often eccentric, ring widths were measured from bark to core alongside the longest radius.

To correct for the presence of missing or false rings, we crossdated the disks both graphically and statistically in the program TSAP-Win version 0.55 (Frank Rinn, Heidelberg, Germany) employing a technique described by Swetnam et al. (1985) and Schweingruber (1989). Grafts were dated with the corresponding corrected root and stem chronologies.

Clone identification—Cambial tissue was excised from the bark samples and was used for DNA extraction using the GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich Canada, Oakville, Ontario, Canada) according to the manufacturer's protocol. Seven microsatellite loci *PTR1*, *PTR2*, *PTR3*, *PTR4*, *PTR5*, *PTR6*, and *PTR8* (Dayanandan et al., 1998; Rahman et al., 2000) were amplified using dye-labeled oligonucleotide primers and *Taq* polymerase (Gibco, Invitrogen Life Technologies, Burlington, Ontario, Canada). Polymerase chain reaction (PCR) was carried out in a 96-Well GeneAmp PCR System 9700 (Applied Biosystems, Foster City, California, USA) in a total volume of 10 μL containing 4 μL DNA extract, 0.625 pmol/μL primers, 0.2 mM dNTP, 3.125 mM $MgCl_2$, 1.4 μL BSA, and 12.5 mM Tris-HCl (pH 8.0). Fragment amplification started with 10 min at 95°C for enzyme activation and DNA strand denaturation, continued with 33 cycles of 1 min denaturation at 95°C, 1 min annealing at touchdown temperatures decreasing by 1°C every cycle from 60°C to 54°C, and 1 min primer extension at 72°C, and terminated with 7 min final extension at 72°C. The PCR product (0.4 μL)was mixed with 0.25 μL internal size standard (TAMRA 500 Liz) and 12 μL deionized formamide, and the mixture was heat-denaturated for 5 min. Fragments were separated by capillary electrophoresis in 3130 Genetic Analyzer (Applied Biosystems). Allele calling was done in GeneMapper version 3.7 (Applied Biosystems).

To determine the resolving power of the set of microsatellites used, we used the round robin method to estimate the probability (p_{gen}) that consecutively sampled trees that actually belong to different clones would, by chance, have a similar genotype (Parks and Werth, 1993):

$$P_{\text{gen}} = \Pi(\sum_{i=1}^{l} f_i g_i) 2^h,$$

where l is the number of loci, h is the number of heterozygote loci, and f and g are the allelic frequencies of alleles f and g. Computation was done in the program GenClone v1.1 (Arnaud-Haond and Belkhir, 2007). Moreover, to screen for possible somatic mutations, PCR artifacts, and scoring errors, the frequency distribution of genetic distances among multilocus genotypes (based on the number of different alleles among sampled trees) was computed in the same program.

RESULTS

Clone identification—In total, 68 ramets were excavated in an area of 88 m^2. Among these, the seven microsatellite markers identified 18 clones, with an average clonal size of 3.8 ramets and a maximum of 13 ramets (Table 1). Thirty-three percent of clones had only one ramet. The resolving power of molecular markers was high. The probability of sampling two trees sharing the same genotype and being derived from a distinct sexual reproductive event, p_{gen}, was lower than 0.01, 0.001, and 0.003 in all the clones in sites W, K, and M, respectively. The frequency

TABLE 1. Clonal characteristics of study sites M, W, and K in northwestern Quebec, Canada.

Characteristic	Site			Total
	M	W	K	
Mean age of ramets	42	39	23	35
No. of ramets	17	18	33	68
No. of ramets per m^2	0.52	0.55	1.5	0.77
No. of clones	5	2	11	18
No. of single-ramet clones	1	0	5	6
Mean no. of ramets per clone	3.4	9	3	3.78
Max. no. of ramets	9	13	11	13

distribution of genetic distances did not indicate any somatic mutations or scoring errors. All clones identified in the three study sites differed by six or more alleles (data not shown).

Natural root grafting—Grafting was frequent, averaging 0.54 grafts per tree and 0.42 grafts per excavated square meter (Table 2). Number of grafts per tree as well as number of grafts per square meter was significantly lower in site M than in site W ($\chi^2 = 9.05$, df = 1, $P < 0.01$; $\chi^2 = 9.8$, df = 1, $P < 0.01$). We found both intra- and interclonal grafts (Table 2). There was no significant difference in the number of these two graft types either when considering both the grafted stems and LRDS together ($\chi^2 = 0.03$, df = 1, $P > 0.85$) or when counting grafts between stems and between stems and LRDS separately ($\chi^2 = 0.43$, df = 1, $P > 0.5$; $\chi^2 = 0.6$, df = 1, $P > 0.4$). More than one third of all grafts connected living stems to LRDS (Table 2). The number of grafts was negatively correlated with the distance between trees ($r^2 = 0.26$, $P = 0.02$). Most grafts were formed in close proximity to stems (Table 2). Average age of roots at the time when the first common ring was created was 20 yr but varied greatly from 2 to 41 yr (Table 2).

Living roots on dead stumps (LRDS)—Excavation uncovered 25 LRDS belonging to 10 different clones. All the stumps, apart from one, had some root connections to at least one living bole. The only unconnected dead ramet whose roots were still alive died during the summer of excavations. Stumps of the majority of LRDS were located directly on living roots and were connected through the surviving parental roots to the living ramets of the same genotype (Fig. 1). Almost half of the LRDS were also grafted to other roots or stems. Sixteen percent of the LRDS were only integrated into the root systems of different clones. All genotypes that were present among the roots were also present among the stems.

Clonal integration—Thirty-eight percent of the ramets were not connected to at least one other living ramet within excavated areas. Parental root connections between at least two living trees were preserved in 32% of stems. The remaining 30% of stems were attached to at least one other living tree only through root grafts. Nevertheless, the percentages of trees interconnected through parental roots and grafts varied greatly among sites (Table 3). Clonal integration was observed in all sites. The root system of the stands in sites W and K, where grafting was more frequent, is simplified in Fig. 2. Two large networks (1 in site K and 1 in site W) connecting different clones, ramets, and LRDS through parental roots and grafts were found. The largest network integrated 10 living trees from four different clones (1, 1, 2, and 6 ramets) and five LRDS from two clones (one and four stumps from each). The second largest root system integrated seven living trees from two clones (two and five ramets) and four LRDS from two clones (two stumps from each).

TABLE 2. Summary of graft incidence, location, and dating of root age when grafting completed (the first common ring formation).

Graft variable	Site			Total
	M	W	K	
No. of grafts	3	17	17	37
No. of grafts/tree	0.176	0.944	0.515	0.544
No. of grafts/m^2	0.091	0.515	0.773	0.420
No. of grafts between different clones	1	10	8	19
No. of grafts within clones	2	7	9	18
No. of grafts between living stems	2	9	10	21
No. of grafts between living stems and LRDS	1	7	7	15
Mean root age when grafting complete	24.5	20.79	19.26	20.03
Max root age when grafting complete	25	32	41	41
Min root age when grafting complete	24	6	2	2
Mean distance between grafted stems (cm)	18	60	33	43
Max distance between grafted stems (cm)	55	120	182	182
Mean distance of grafts to stems (cm)	10	38	15	25
Max distance of grafts to stems (cm)	27	120	182	182

DISCUSSION

This study is the first to show that ramets of different aspen clones can be integrated through root grafts into a communal root system. Even though we had anticipated finding grafts established between clones, basing our clone conservation hypothesis on their presence, we were surprised to discover that, in fact, interclonal grafting in our sites was just as frequent as intraclonal grafting. A greater degree of genetic similarity was predicted to lead to more grafting because grafts within the same genotype (within a single tree) are far more common than between genotypes (Loehle and Jones, 1990). Moreover, grafting was reported to be especially frequent in species known for genetic uniformity (Stone, 1974). So how do we explain a high frequency of interclonal grafting in such a diverse species as trembling aspen?

Two factors may contribute to this observation: a high density and proximity of aspen ramets in young stands and aspen's clonal morphology. This study (Table 2) as well as reports of other authors investigating natural root grafting in trembling aspen and other tree species (Eis, 1972; Gordon and Roth, 1976; DesRochers and Lieffers, 2001) showed that tree proximity is an important factor influencing the probability of graft formation. Because grafting requires a physical contact of roots, a close tree and high root density may lead to an increased frequency of grafting. Moreover, aspen clones form rather loose clusters in which ramets from different clones are often spatially mixed (Namroud et al., 2005). Therefore, a high root density and clones' intermingling may cause more frequent contact between roots of different genotypes and may thus result in a more frequent interclonal grafting in contrast to other tree species. For instance, roots of northern prickly ash cease growth as soon as they start approaching roots of different genotypes, making interclonal grafting virtually impossible (Reinartz and Popp, 1987). Nonetheless, in trembling aspen, genetic similarity and between-individual chemical differences seems to bear much less importance for the probability of grafting than previously suggested.

Conservation of root segments of different genotypes via root grafts to living ramets presumes not only grafting between different clones but also its frequent occurrence. Indeed, interclonal grafting was frequent in two of the three sites in this study. The overall graft incidence ranged around 0.5 grafts per tree and was similar to observations in aspen stands in Alberta (DesRochers and Lieffers, 2001). These findings contrast with older reports that natural root grafting rarely occurred in aspen (Barnes, 1966; Shepperd, 1993). Some grafts in these studies

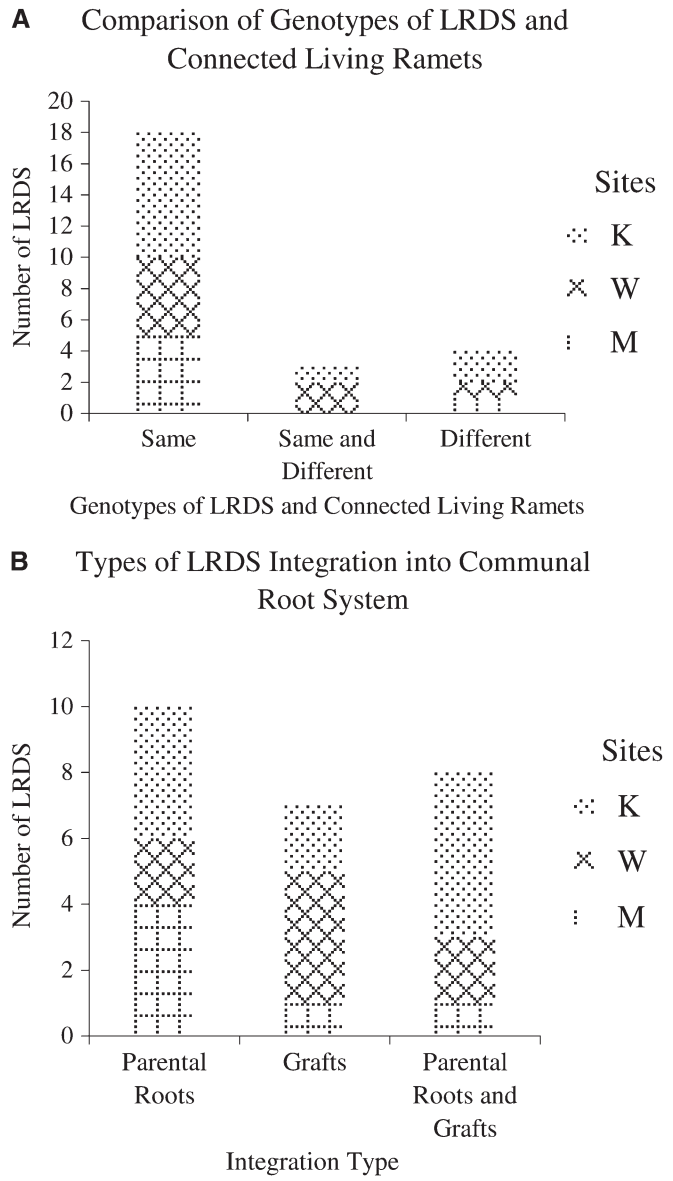

Fig. 1. (A) Genotype comparison between ramets of *Populus tremuloides* and living roots on dead stumps (LRDS) and (B) their type of connection at three sites in northwestern Quebec, Canada.

could have been overlooked; none of them employed either dendrochronological analysis or clone identification, which helps differentiate grafts established close or directly on stumps from original parental root connections. Moreover, grafting frequencies in these studies might have been underestimated due to partial excavations. More than one third of all the grafts we found were located between living trees and LRDS (Table 2), and some of these would have gone undiscovered if we had only partially excavated the living stems.

However, even among our sites, there was some variation in graft incidence. In particular, grafts were scarcer in site M than in site W despite otherwise similar age and stem densities. This difference might be caused by other site characteristics such as rooting depth known to influence graft formation (Bormann and Graham, 1959; Eis, 1972). The deeper the soil, the lower is the resulting root density, which in turn gives roots fewer chances to cross and form grafts. Moreover, other factors, such as exposure of sites to wind, that are believed to facilitate graft initiation due to root abrasions might be involved. Nevertheless, the causes of grafting are still poorly understood and their investigation was far beyond the scope of this study.

The dendrochronological reconstruction showed that graft formation started early after stand initiation, and even trees in the youngest stand (23 yr old) were commonly interconnected through root grafts (Table 2). The minimum root age of 2 and 6 yr at the time of the first common growth ring formation indicated that the process of grafting may not take very long to be completed. The large variation in the timing of graft completion pointed to the fact that grafting was not restricted to a particular period but rather continued throughout the life of stands. Hence, there should be more grafts in old stands than in young, yet a comparison of our young and middle-aged sites with old, declining aspen stands (DesRochers and Lieffers, 2001) failed to demonstrate such a difference. An increased graft incidence in old sands may be buffered by decreasing stem density and loss of some grafts during the stand aging. If this is true, graft loss in old stands may pose a constraint to the potential contribution of grafting to clonal diversity maintenance in this species.

The root system excavation did not uncover any "hidden" genotypes that would not be present in the aboveground part of the stands. Most LRDS were connected by parental roots to living ramets of the same genotype (Fig. 1) and were, therefore, likely to be a result of self-thinning, a process of density-dependent mortality in young stands (Johnstone et al., 2004). Nevertheless, some LRDS had no parental connections and continued to live only via interclonal grafts (Fig. 1). Provided these stumps stay alive until the next disturbance, root suckers of these clones could appear even in places where there were no living stems of the corresponding genotypes before the disturbance.

Our concept of clone conservation in a form of LRDS largely depends on the span of time over which LRDS can stay alive. The longer their lifespan is, the better the chances are for the clone survival. Roots on dead stumps of Douglas-fir (*Pseudotsuga menziesii*) were still alive 32 yr after selective cuttings (Eis, 1972). Graham and Bormann (1966) reported in their review continued growth of LRDS in more than 50 tree species. The oldest one persisted for as long as 84 yr. The timing of the death of LRDS in this study could not be determined because of the completely or partially rotten cores that typically left only a hole in the place of a stem. Nevertheless, it is tempting to speculate that they can stay alive for many years provided the supply of carbohydrates from the living tree remains uninterrupted.

Clone survival would also be favored by a large size of the communal root system in which roots of different clones could be "captured." Integration of trees into a communal root system in our young and middle-aged stands as well as in declining aspen stands in Alberta was high, leaving only a few unconnected individuals (DesRochers and Lieffers, 2001). The percentage of independent trees is likely to be overestimated; the

TABLE 3. Integration type of aspen ramets into the communal root system.

Connection	Site			Mean
	M	W	K	
Parental roots only (%)	67	0	32	33
Parental roots and grafts (%)	0	9	36	19
Grafts only (%)	33	91	32	48

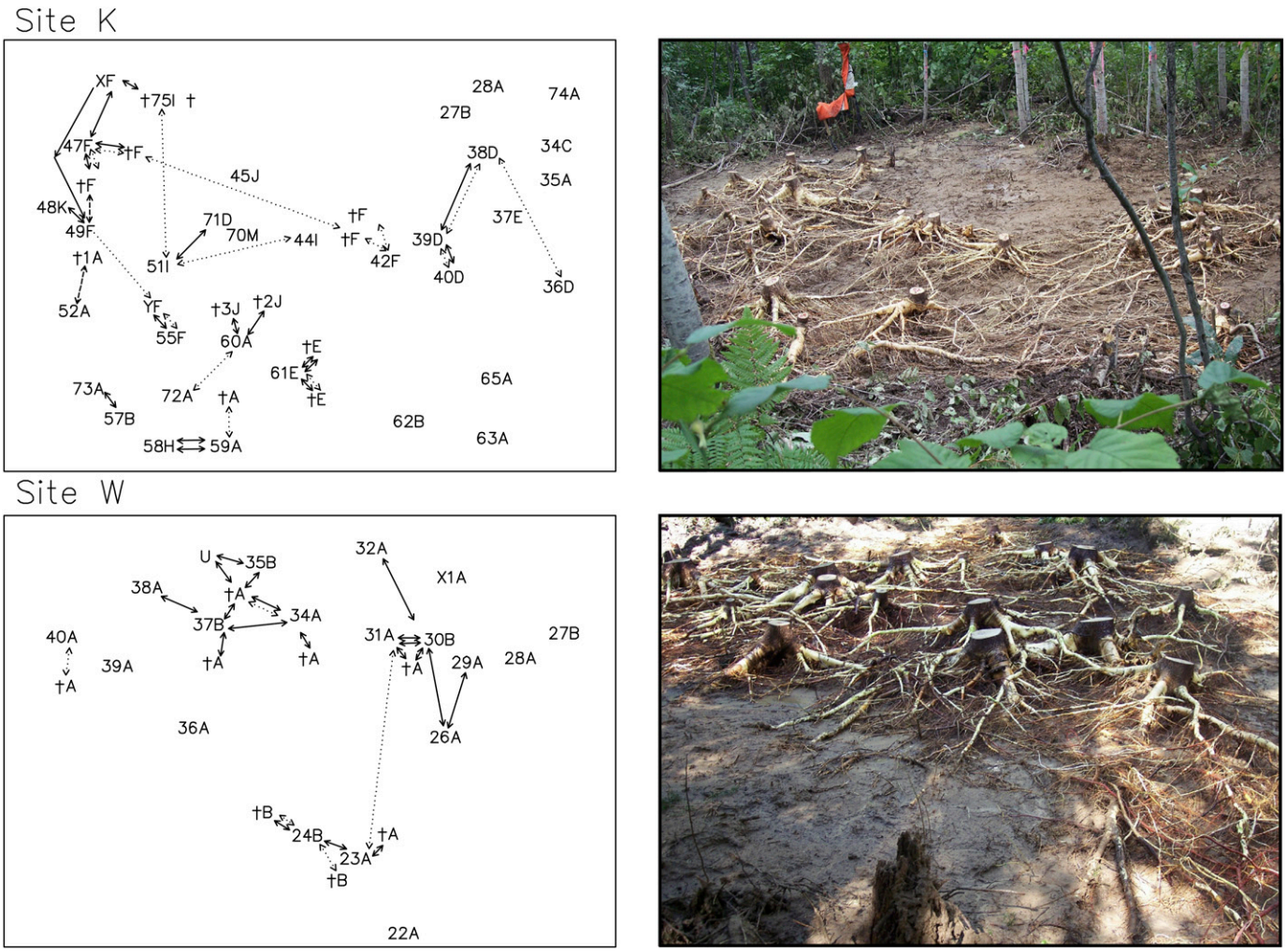

Fig. 2. Schemas and photographs of root systems of *Populus tremuloides* and clonal integration at sites W and K in northwestern Quebec, Canada. Living ramets are numbered, clones are distinguished by different letters, and living roots on dead stumps (LRDS) are marked by a dagger (†) before the clonal identification. Parental roots and grafts are depicted by dotted and solid lines, respectively. Dashed line at left in site K shows roots connecting two individuals that could not be dated because of broken or rotten cores.

excavated plots in this and other studies were relatively small, and some trees could have been connected to trees outside the plots. This explanation was also likely for the substantial variation among the sites in the proportion of trees linked by parental roots (Table 3). In particular, site W had an obvious lack of parental root connections although the parental roots were preserved in all the trees of this site and their proportion to new roots was comparable with the other stands (data not shown). Therefore, we assume that the lack of parental root connections in this site was an artifact caused by a coincidence of the shape of the excavated area and the shape of clones, which tend to be rather irregular due to a "guerrilla" growth type of aspen (Namroud et al., 2005).

In the current study, we have not attempted to exhaustively explain the complex phenomenon of diversity maintenance in clonal species; rather we have tried to suggest an additional mechanism that could be involved in diversity maintenance in trembling aspen. We have not found an unassailable evidence to support our "genotype bank" hypothesis. Nevertheless, this study showed that grafting in aspen can be extensive regardless of the root genotype and that there is a substantial interconnectivity among aspen trees both through surviving parental roots and root grafts. From this perspective, aspen stands should be seen as large, genetically diverse physiological units rather than discrete individuals or genetically uniform clones. Even though the root systems that we uncovered did not include roots of unique genotypes, acquiring roots of dead trees either through parental roots or root grafts helps to maintain extensive root systems, which is crucial for clone survival (Pelton, 1953; Tappeiner et al., 1991). Finally, excavation of larger plots in late-successional stands with only a few aspen trees per hectare left would probably have better chances of discovering unique genotypes to finally validate or reject our hypothesis.

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