MUTATION LOAD IN NATURAL POPULATIONS OF THE SENSITIVE FERN
*ONOCLEA SENSIBILIS* EXPOSED TO SOIL MUTAGENS

DANIEL J. SCHOEN, JOHN S. BROWNSTEIN,1 AND PAUL A. WHITE2

Department of Biology, McGill University, 1205 Avenue Docteur Penfield, Montreal, Quebec H3A 1B1 Canada

Abstract. This study examined mutations expressed in haploid fern gametophytes derived from spores collected in the wild in order to assay for the presence of mutagenic compounds in natural environments. Gametophytes of the fern *Onoclea sensibilis* were cultured from spores collected in 14 natural populations around the region of Montreal, Quebec, Canada. Most of the populations grew in soils exposed to spring flooding of the St. Lawrence River or its tributaries. Relative variation in the rate of somatic mutation was calculated from the proportion of fern clones in each population segregating for mutations that disrupt gametophyte development. Estimated somatic mutation rates ranged over approximately one order of magnitude among populations, with most of the variation due to differences between populations located downstream from Montreal (higher mutation rates) vs. those upstream or outside the immediate vicinity of the river (lower mutation rates). Organic (dichloromethane) extracts of soil samples from 11 of these sites were examined for mutagenicity using the plate incorporation version of the Salmonella/mammalian microsome test (strains TA98, TA100, YG1041, YG1042). Salmonella mutation rates per milligram equivalent of dry soil (i.e., mutagenic potency) were roughly an order of magnitude greater for soils from downstream vs. upstream locations. TA98 (frameshift) mutagenicity of soil extracts is positively correlated with the estimated proportion of the fern population segregating for gametophytically expressed mutations. The pattern of Salmonella mutagenicity results suggests the presence of homocyclic polycyclic aromatic hydrocarbons (PAHs). Results are discussed with respect to population viability of the fern *O. sensibilis*, the ability of microbial assays to identify mutagenic hazards to natural populations, previous studies of genotoxic pollution in the St. Lawrence River system, and previous studies of soil mutagenicity.

Key words: conservation genetics; environmental mutagenesis; mutational meltdown; soil mutagens; St. Lawrence River.

INTRODUCTION

Research investigating the effects of pollution on the viability of naturally occurring plants and animals has frequently focused on acute physiological responses. Acute toxicity is readily detected by its rapid effects on exposed organisms. Exposure levels are usually high and the effects are generally severe (e.g., death). While these are cause for concern, other less easily detected effects of environmental pollution that can occur at far lower exposure levels may be of equal or greater importance. Although these effects are not manifested as an acute lethal response, they can reduce the long-term viability of exposed organisms through chronic effects (e.g., chronic tissue damage, neoplasia, etc.), and/or reduce the viability of natural populations through reductions in reproductive success and effects on particularly sensitive life cycle stages (e.g., embryos and juveniles). For example, environmental pollution may bring about reductions in reproductive fertility that lead to declines in growth rates of populations that become manifest only after a number of generations (i.e., once population decline or limited recruitment into younger age classes becomes apparent). Even more difficult to detect are chronic effects caused by deleterious mutations, particularly those that impact the germ lines of exposed organisms. The accumulation of deleterious mutations in the gene pool of a population increases its genetic load (Muller 1950). This may lead to elevated probabilities of local extinction, either through the direct, fitness-reducing effects of the mutations themselves, or due to increased levels of inbreeding depression (Frankham 1995). Lynch et al. (1995a, b) developed demographic models that assume background deleterious mutation rates of approximately one mutation per genome per generation, as inferred from natural populations of *Drosophila melanogaster* (Mukai 1964, 1979). In these models, they hypothesized a connection between population growth rate and mutation load and showed that populations that have become reduced in effective size may be subject to increased risk of extinction through mutation accumulation. They point out that if deleterious mutation rates are greater than assumed in their analyses (e.g., due to...
the release of mutagenic pollutants), the extinction probabilities due to mutation accumulation under their model could be correspondingly higher.

The detection of mutagenic hazards in natural environments requires tools for rapid and effective detection of mutagenicity. This need has led to the development of a wide range of laboratory bioassays that employ cells in culture (e.g., bacteria, yeast, and animal cells) or experimental organisms (e.g., rodents, insects, plants) to detect genotoxic responses following exposure to contaminated environmental media (e.g., soil, sediments, water, airborne particulates), organic extracts of contaminated environmental media, or individual substances that are suspected mutagens (e.g., pyrogenic polycyclic aromatic hydrocarbons; Lewtas 1991, Li and Loretz 1991). The in vitro assays employing bacteria, yeast, or animal cells exposed in liquid suspension are by far the least expensive and most convenient assays. The most popular in vitro assay is the Salmonella/mammalian microsome assay, more commonly referred to as the Salmonella mutagenicity test or simply “the Ames test” (Ames et al. 1973, Maron and Ames 1983). The test detects the reversion of histidine-requiring auxotrophs of Salmonella typhimurium to prototrophy following exposure to compounds or complex mixtures. Since bacteria cannot enzymatically transform many mutagens into the potent electrophiles that interact directly with DNA, exogenous metabolic activation mixtures are often used to simulate the in vivo metabolism and activation of potential mutagens referred to as promutagens. While exogenous activation mixtures from many organisms have been used to augment the Salmonella mutagenicity test (e.g., fish, humans, plants, rodents), the most popular activation mixture is the 9000-g supernatant of livers from Aroclor 1254-induced male rats, often referred to simply as S9.

Although short-term microbial bioassay such as the Salmonella mutagenicity test can provide sensitive and rapid means for the detection of mutagenic hazard in natural environments, extrapolation of the results to wild populations is problematic for a number of reasons. Wild populations of plants and animals experience long-term exposure to mutagenic substances and may respond to lower concentrations of mutagens as compared with bacteria exposed in vitro. In addition, since wild organisms pass on genes to future generations, deleterious mutations may accumulate in the populations and augment damage caused by ongoing genotoxic pollution. Moreover, wild organisms may possess physical barriers to mutagenic pollutants, detoxification mechanisms, and/or DNA repair pathways that reduce the likelihood of mutagenic effects being manifested in situ. Therefore, although environmental testing with microbial assays can reveal the presence of genotoxic compounds, it remains unclear how the long-term viability and genetic composition of natural populations correlate with results obtained using these hazard identification tools. Therefore, an assessment of natural population viability in response to mutagenic contamination requires both in vitro hazard identification as well as subsequent in situ effect assessment.

Effective assessment of in situ mutagenic effects on wild organisms has proved to be an exceptional challenge. The most popular techniques assess the frequency of DNA damage events and/or cytogenetic abnormalities in wild organisms such as rodents (e.g., McBee et al. 1987), fish (e.g., diGiulio et al. 1993, Belpaeme et al. 1998), invertebrates (Nacci et al. 1992, Hutchinson et al. 1998, Mitchelmore and Chipman 1998), and plants (e.g., Klekowski and Berger 1976, Batalha et al. 1999, Kong and Ma 1999) collected at contaminated sites or placed at contaminated sites for a specified time period. Obvious technical difficulties have hindered attempts to assess the actual frequency of mutations at a given locus or series of loci in wild organisms. In the 1970s, Klekowski and colleagues (Klekowski 1973, 1976, Klekowski and Berger 1976, Klekowski and Levin 1979, Klekowski and Klekowski 1982) pioneered the use of ferns as in situ monitors of mutagenic pollution. Ferns possess a number of advantages for the detection of environmentally induced mutations. Two of these, in particular, are relevant to the present study. First, as discussed by Klekowski and Berger (1976), the cells in the body of the fern are derived from a single apical meristematic cell located at the tip of the growing rhizome (Wardlaw 1967). A mutation arising in this apical cell is passed on somatically to the daughter cells, including those that differentiate into spore mother cells. Thus, when haploid spores are produced by meiosis, a 1 : 1 ratio of mutation-bearing to wild type spores is expected at the affected loci. Second, germination of the haploid spore leads to a free-living haploid gametophyte with a distinct developmental morphology. Developmental studies have indicated that while the early-developing gametophyte may rely upon gene products passed on from the spore mother cell, the later developing gametophyte is metabolically independent (Klekowski and Klekowski 1982). If the spore inherits a deleterious mutation (e.g., one that inhibits gametophyte metabolism), the gametophyte may undergo only a few cell divisions before it aborts. This leads to an expected 1 : 1 ratio of aborted (mutation-possessing) to normal gametophytes. Klekowski and Klekowski (1982) demonstrated the utility of this in situ assay by studying a fern population located near an electrical transformer manufacturer in the state of Massachusetts, USA. It had been suspected that the population in question was exposed to mutagenic pollutants, and his test results revealed an elevated incidence of mutation.

Previous investigations of St. Lawrence River system in Quebec, Canada, have revealed that mutagenic wastewaters enter the river from a wide range of industrial (e.g., pulp and paper mills, metal refining and founding facilities, organic chemical production facil-
TABLE 1. Locations and descriptions of sampled Onoclea sensibilis populations in Quebec, Canada.

<table>
<thead>
<tr>
<th>Population</th>
<th>Location relative to Montreal</th>
<th>Latitude, longitude</th>
<th>Site description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baie-du-Febvre,</td>
<td>downstream</td>
<td>46°09’ N, 72°45’ W</td>
<td>marsh with low willows</td>
</tr>
<tr>
<td>Sorel</td>
<td>downstream</td>
<td>46°01’ N, 73°07’ W</td>
<td>along shoreline in wet sand</td>
</tr>
<tr>
<td>Berthierville</td>
<td>downstream</td>
<td>46°01’ N, 73°10’ W</td>
<td>along shoreline in wet soil</td>
</tr>
<tr>
<td>Lanoraie</td>
<td>downstream</td>
<td>46°00’ N, 73°11’ W</td>
<td>grassy marsh</td>
</tr>
<tr>
<td>Contrecoeur</td>
<td>downstream</td>
<td>45°50’ N, 73°14’ W</td>
<td>wet soil at river edge</td>
</tr>
<tr>
<td>Lavaltrie</td>
<td>downstream</td>
<td>45°55’ N, 73°17’ W</td>
<td>Typha marsh</td>
</tr>
<tr>
<td>Repentigny</td>
<td>downstream</td>
<td>45°44’ N, 73°26’ W</td>
<td>marsh</td>
</tr>
<tr>
<td>Dollard des Ormeaux</td>
<td>upstream</td>
<td>45°30’ N, 73°49’ W</td>
<td>wet soil at river edge</td>
</tr>
<tr>
<td>Senneville</td>
<td>upstream</td>
<td>45°26’ N, 73°57’ W</td>
<td>marsh</td>
</tr>
<tr>
<td>Saint-Anicet</td>
<td>upstream</td>
<td>45°07’ N, 74°21’ W</td>
<td>marsh</td>
</tr>
<tr>
<td>Waterloo</td>
<td>away from St. Lawrence</td>
<td>45°21’ N, 72°31’ W</td>
<td>wet forest</td>
</tr>
<tr>
<td>Mont St. Hilaire</td>
<td>away from St. Lawrence</td>
<td>45°33’ N, 73°10’ W</td>
<td>wet forest</td>
</tr>
<tr>
<td>St. Bruno</td>
<td>away from St. Lawrence</td>
<td>45°33’ N, 73°19’ W</td>
<td>wet forest</td>
</tr>
<tr>
<td>Covey Hill</td>
<td>away from St. Lawrence</td>
<td>45°01’ N, 73°45’ W</td>
<td>wet forest edge</td>
</tr>
</tbody>
</table>

Ities) and municipal sources (White et al. 1996). Additional analyses indicated that areas immediately downstream from the city of Montreal are appreciably more contaminated than those located upstream (White and Rasmussen 1998). The contrast between these regions provides an ideal opportunity for in situ validation of the presumed mutagenic hazard. Here we report the results of an assessment of the regional variation in mutation frequencies in wild populations of the native fern Onoclea sensibilis growing within a 100 km radius of the city of Montreal, Quebec, Canada. Specimens were collected from soils exposed to the waters of the St. Lawrence River and its tributaries and in four populations growing outside the immediate vicinity of the river. The geographically varying pattern of mutation frequencies detected in the fern populations is compared with Salmonella mutagenicity results on organic soil extracts sampled from the sites where the ferns grew, and the consequences of the findings are interpreted with respect to population viability and extinction risk in Onoclea sensibilis.

METHODS

Study organism and population sampling

Onoclea sensibilis, the sensitive fern, occurs naturally in swamps, wet forests, and riparian habitats throughout the northeastern United States and adjacent Canada. The populations are composed of individuals that propagate clonally from rhizomes that grow beneath the surface of the soil. The rhizomes frequently are interdigitated, and so separate genets are difficult to distinguish spatially from one another. The plants produce dimorphic fronds that are either sexual (sporangia-bearing) or vegetative (sterile). Populations typically consist of thousands of vegetative fronds. In Quebec, sporangia mature in mid-September, and the peak of spore dehiscence occurs in October.

Fourteen populations were sampled (Table 1). Ten of these were located along the banks of the St. Lawrence River or its immediate tributaries in Quebec and were at least 10 km apart from one another; three populations are upstream and seven are downstream from the city of Montreal. Another four populations were sampled from locations outside the immediate vicinity of the river (Fig. 1). Plants in the riverside populations grew in damp soils located inside the spring flood zone, as evidenced by their position relative to the high water mark that is apparent from the deposition of floating debris left behind by the river.

In October of 1998, 20 sexual fronds were collected at random from each population, under the constraint that none be located <5 m from any other collected. This was done to increase the number of individual genets sampled. Fronds were collected before spore release began and in the laboratory were wetted by immersing them overnight in distilled water. Excess water was removed and each frond was then transferred to a separate glassine bag. The fronds were dried in an incubator overnight at 30°C to promote spore release. Spores that collected at the bottoms of the bags were stored at room temperature for 2–4 wk before being used in studies of gametophyte growth and development.

Gametophyte culture and scoring

Spores from each frond were sown separately in 150 × 15 mm petri dishes containing 20 mL of inorganic salt medium (Murashige and Skoog modified basal salt mixture without sucrose; Sigma Chemical Company, St. Louis, Missouri, USA) solidified with 1% agar. Spores from each glassine bag were shaken lightly over the agar surface of a single petri dish such that the density per dish ranged from ~200 to 500 spores. The sowing of spores was done under a laminar flow hood. Because of the large number of samples processed, spores were not surface sterilized (<5% of cultures developed fungal or bacterial growth, as judged by the presence of fungal hyphae or bacterial colonies on the plates, and heavily contaminated plates were discarded). Direct sowing of spores without surface sterilization is suggested when rapid, synchronous spore germination is important (Dyer 1979:283). Immediately
following sowing, the petri dishes were transferred to a tissue culture room and placed under 120 μmol·m⁻²·s⁻¹ of continuous florescent illumination at 25°C. In previous studies, light has been demonstrated to stimulate germination of Onoclea spores (Wayne and Hepler 1984).

After 4 wk, the gametophyte cultures were examined at 20–30× magnification using a compound dissecting microscope. Beneath each dish, we placed a sampling grid composed of 1 × 1 cm squares. Squares in the grid were selected at random until each dish yielded a sample of 100–150 gametophytes. In ∼10% of the dishes, spore samples were small or there was localized contamination, and <100 gametophytes were examined. All gametophytes within the selected squares were characterized morphologically. The most commonly observed gametophyte morphology was the normal, cordate type (Fig. 2). Several aberrant types of gametophytes were also observed. Among these we distinguished three: aborted, callus, and achlorophyllous. Aborted gametophytes were the most common aberrant type. They consisted of several green prothallial cells (i.e., the result of two to four mitoses) and a short rhizoid (Fig. 3). The callus type was much more rare. It lacked the organized cordate structure of the normal gametophyte and instead took on various multilobed forms. The achlorophyllous gametophytes de-
veloped near-normal morphology but were pale green or completely lacking in visible chlorophyll (not illustrated).

Soil sampling and Ames testing of soil extracts

A 10 cm deep × 1 cm diameter soil core was sampled from the center of 11 populations. Soil samples were stored frozen at −20°C prior to extraction. Soil samples were dried with an equal mass of anhydrous sodium sulfate and Soxhlet extracted (1–3 g dry mass) for 16 h with pesticide grade dichloromethane. Subsequent sample cleanup employed gel permeation chromatography on Biobeads SX-3 (Biorad Laboratories, Mississauga, Ontario, Canada). Details of the extraction and cleanup methodology can be found in USEPA (1986), Czuczwa and Alford-Stevens (1989), and White et al. (1998a). Final extracts were stored in 1 mL of dichloromethane and exchanged for 1 mL of high-purity dimethyl sulfoxide prior to mutagenicity testing. Mutagenicity of soil extracts was assessed using the standard plate incorporation version of the Salmonella/mammalian microsome assay (see Maron and Ames 1983 for details). Initial experiments employed four strains of *Salmonella typhimurium*, the standard frameshift and base-pair (i.e., transition and transversion) mutation strains TA98 (*hisD3052, rfa, ΔuvrB, pKM101*) and TA100 (*hisG46, rfa, ΔuvrB, pKM101*), as well as metabolically enhanced versions of TA98 and TA100 known as YG1041 and YG1042 (see Hagiwara et al. 1993 for details). The latter strains produce enhanced levels of O-acetyl transferase (OAT) and nitroreductase (NR) that permit the detection of aromatic amines (in the presence of S9) and nitroarenes (in the absence of S9), respectively. All tests were carried out in duplicate both in the presence and the absence of a metabolic activation mixture containing S9 from Aroclor 1254 induced male rat livers (30 μL S9 per plate; S9 from Molecular Toxicology, Boone, North Carolina, USA). Bacterial strains were graciously provided by Dr. David DeMarini, Environmental Carcinogenesis Division, US EPA, Research Triangle Park, North Carolina, USA. Maximum test concentrations ranged from 120 to 678 mg dry mass equivalents per plate, depending on sample potency and availability. Mutagenic potency values, the slope of the initial linear portion of the concentration–response function, were calculated using ordinary least squares linear regression. A significant positive response was defined as a well-behaved concentration–response function with a mutagenic response exceeding twice the mean control value (i.e., solvent blank). Quality controls included routine examination of master plates and frozen permanent cultures for required genetic markers (Maron and Ames 1983). In addition, several reference mutagens such as sodium azide, 2-nitrofluorene, and 2-aminoanthracene (Aldrich Chemicals, Milwaukee, Wisconsin, USA) were routinely used as positive controls.

RESULTS

Regional variation in overall proportions of aberrant gametophytes observed

In general, at least one ramet in each population produced spores that gave rise to gametophytes with aberrant morphology. Total frequencies of aberrant gametophytes were highest in populations located along the St. Lawrence River and downstream from Montreal. The total frequency of aberrant gametophytes averaged nearly 40% in this region (Table 2). The vast majority of the aberrant gametophytes (>99%) exhibited the “aborted gametophyte” morphology. These were observed in intermediate proportions in populations located upstream from Montreal and in smaller proportions in populations located away from the river (Table 2).

Nested analysis of variance on the angular-transformed proportions of aberrant gametophytes sampled from the sexual fronds in each of the 14 populations indicated significant interregional (downstream, upstream, and nonriver) and intraregional variation (Table 3). Variance component analysis shows that 46% and 33%, respectively, of the overall variance in proportion of aberrant gametophytes is associated with these sources.
TABLE 2. Total frequency of aberrant gametophytes observed in different populations of *Onoclea sensibilis*.

<table>
<thead>
<tr>
<th>Population</th>
<th>Frequency of aberrant gametophytes†</th>
<th>Number of ramets in sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Downstream</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baie-du-Febvre</td>
<td>0.37</td>
<td>20</td>
</tr>
<tr>
<td>Sorel</td>
<td>0.34</td>
<td>16</td>
</tr>
<tr>
<td>Berthierville</td>
<td>0.36</td>
<td>20</td>
</tr>
<tr>
<td>Lanoraie</td>
<td>0.36</td>
<td>20</td>
</tr>
<tr>
<td>Contrecoeur</td>
<td>0.48</td>
<td>20</td>
</tr>
<tr>
<td>Lavaltrie</td>
<td>0.44</td>
<td>16</td>
</tr>
<tr>
<td>Repentigny</td>
<td>0.39</td>
<td>19</td>
</tr>
<tr>
<td>Upstream</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dollard des Ormeaux</td>
<td>0.41</td>
<td>19</td>
</tr>
<tr>
<td>Senneville</td>
<td>0.17</td>
<td>19</td>
</tr>
<tr>
<td>Saint-Anicet</td>
<td>0.21</td>
<td>20</td>
</tr>
<tr>
<td>Away from river</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Waterloo</td>
<td>0.13</td>
<td>20</td>
</tr>
<tr>
<td>Mont St. Hilaire</td>
<td>0.20</td>
<td>16</td>
</tr>
<tr>
<td>St. Bruno</td>
<td>0.31</td>
<td>20</td>
</tr>
<tr>
<td>Covey Hill</td>
<td>0.11</td>
<td>18</td>
</tr>
</tbody>
</table>

† (Number of aberrant gametophytes observed)/(total number of gametophytes assayed).

Estimation of the proportion of individual plants segregating for single-locus mutations

Within populations, the production of aberrant gametophytes was nonrandomly distributed with respect to the individual ramets sampled. Specifically, we observed a bimodal distribution of aberrant gametophytes, in which one mode was centered on the proportion of 0.5 aberrant (0.5 normal), while the second fell at −0.2 aberrant (0.8 normal) gametophytes (Fig. 4). This distribution departs from what would be expected under the purely genetic hypothesis that aberrant gametophytes arise only because of segregating single-gene mutations in parent (sporophyte) plants. If this were the case, one would expect peaks to be centered on proportions of 0.5 aberrant and 0 aberrant gametophytes. Accordingly, it was hypothesized that apart from being the product of gametophytically expressed mutation, some of the aberrant gametophytes could have been produced by late-germinating or immature (but mutation-free) spores. Thus, the potential for misclassification of gametophytes was incorporated into the procedure used to estimate the proportion of parents segregating for deleterious mutations. Specifically, it was assumed that the production of an aberrant gametophyte by a parent could be attributed to: (1) single-gene mutations within the apical cell that produced the frond and gave rise to the sporogenous tissue; and (2) misclassification of immature or late-germinating spores as mutation-bearing gametophytes. Consequently, the likelihood that the \(i\)th clone is segregating for a gametophyte-disrupting mutation was calculated as:

\[
L_{i, \text{segregating}} = K(0.5 - c)^{n_i}(0.5 + c)^{n_{i,\text{segregating}}}
\]

where \(K\) is a constant (the binomial coefficient), \(n_i\) and

FIG. 4. Distribution of proportions of aberrant gametophytes produced by individual ramets (data from seven downstream populations of *Onoclea sensibilis*).
Table 4. Maximum-likelihood estimates of the proportion of clones within populations of *Onoclea sensibilis* segregating for single-locus mutations (*p*), the probability of observing aberrant gametophyte morphology due to misclassification (*c*), and the somatic mutation rate (*u*).

<table>
<thead>
<tr>
<th>Population</th>
<th>p (95% CI)†</th>
<th>c (95% CI)†</th>
<th>u±</th>
</tr>
</thead>
<tbody>
<tr>
<td>Downstream</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baie-du-Febvre</td>
<td>0.47 (0.16–0.68)</td>
<td>0.12 (0.06–0.32)</td>
<td>0.63</td>
</tr>
<tr>
<td>Sorel</td>
<td>0.57 (0–0.88)</td>
<td>0.09 (0.03–0.39)</td>
<td>0.84</td>
</tr>
<tr>
<td>Berthierville</td>
<td>0.25 (0.05–0.80)</td>
<td>0.24 (0.03–0.32)</td>
<td>0.29</td>
</tr>
<tr>
<td>Lanoraie</td>
<td>0 (0–0.85)</td>
<td>0.36 (0.05–0.42)</td>
<td>0.73</td>
</tr>
<tr>
<td>Contrecoeur</td>
<td>0.75 (0.10–0.90)</td>
<td>0.12 (0.05–0.46)</td>
<td>1.39</td>
</tr>
<tr>
<td>Lavaltrie</td>
<td>0.42 (0.13–0.69)</td>
<td>0.18 (0.14–0.26)</td>
<td>0.58</td>
</tr>
<tr>
<td>Repentigny</td>
<td>0.43 (0.21–0.74)</td>
<td>0.14 (0.09–0.28)</td>
<td>0.63</td>
</tr>
<tr>
<td>Upstream</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dollard des Ormeaux</td>
<td>0.05 (0–0.16)</td>
<td>0.38 (0.33–0.42)</td>
<td>0.05</td>
</tr>
<tr>
<td>Senneville</td>
<td>0.05 (0–0.31)</td>
<td>0.13 (0.06–0.18)</td>
<td>0.05</td>
</tr>
<tr>
<td>Saint-Anicet</td>
<td>0.25 (0–0.50)</td>
<td>0.11 (0.05–0.22)</td>
<td>0.29</td>
</tr>
<tr>
<td>Away from river</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Waterloo</td>
<td>0 (0–0)</td>
<td>0.13 (0.10–0.17)</td>
<td>0.05</td>
</tr>
<tr>
<td>Mont St. Hilaire</td>
<td>0.06 (0–0.31)</td>
<td>0.18 (0.10–0.26)</td>
<td>0.06</td>
</tr>
<tr>
<td>St. Bruno</td>
<td>0.15 (0–0.70)</td>
<td>0.23 (0.05–0.30)</td>
<td>0.16</td>
</tr>
<tr>
<td>Covey Hill</td>
<td>0.06 (0–0.17)</td>
<td>0.08 (0.06–0.11)</td>
<td>0.06</td>
</tr>
</tbody>
</table>

† Sample sizes (number of fronds per population) are given in Table 2. Confidence intervals were obtained by bootstrapping (see Results: Estimation of the proportion of individual plants. . .).

‡ Per unit of vegetative growth. Estimated variation in *u* is in relative terms, as clone size is undetermined (see Results: Estimation of the proportion of individual plants. . .).

*n* are the numbers of normal and aberrant gametophytes in the spore sample observed from the *i*th segregating clone, and *c* is the probability of observing the aberrant gametophyte morphology due to spore immaturity or late germination. The likelihood that the *i*th clone lacks a gametophyte-disrupting mutation, i.e., that it is nonsegregating, was calculated as:

\[
L_{i,\text{nonsegregating}} = K(1 - c)^{n_i}(c)^{2n_i}
\]

where *n* and *n* are the numbers of normal and aberrant gametophytes in the spore sample observed from the *j*th nonsegregating clone. We wished to estimate the number of segregating clones (*N*seg) and nonsegregating clones (*N*nonseg) in each population sample. This was done by maximizing the likelihood:

\[
L(N_{\text{seg}}, N_{\text{nonseg}}, \text{assigned}) = \prod_{i=0}^{N_{\text{seg}}} \prod_{j=0}^{N_{\text{nonseg}}} L_{i,\text{seg}} L_{j,\text{nonseg}}
\]

where maximization was carried out numerically under all possible assignments of the different clone spore samples as deriving from either segregating or nonsegregating clones, jointly combined with values of *c* ranging from 0 to 1.00 (at intervals of 0.01). From this we estimated the parameter *p* = *N*seg/(*N*seg + *N*nonseg), the fraction of the sporophyte population segregating for mutations. The 95% confidence intervals of our estimates of *p* and *c* were obtained by bootstrapping (Efron and Tibshirani 1993) using samples from single clones as the unit of observation. The original data from each population were resampled 500 times, and each time the maximum-likelihood estimates of *p* and *c* were retained. The distributions of *p* and *c* were truncated at the upper and lower 2.5% levels to obtain 95% confidence intervals.

Table 4 shows the estimates for the populations grouped by region. Because the sample sizes (number of clones sampled per population) were relatively small (maximum of 20 clones), the confidence limits around our estimates of *p* and *c* tend to be large. Nevertheless, a clear regional pattern emerges, with the downstream sites having the largest estimated *p* values. These range from *p* = 0 to 0.75 (0.52 ± 0.09, mean ± 1 se). Most estimates have confidence limits that do not overlap 0 (Table 4). In contrast, the *p* values upstream (0.12 ± 0.07) and away from the river (0.07 ± 0.03) are smaller, and all have confidence limits that overlap 0 (Table 4). The estimates of *c* show no pronounced regional variation, and most fall between 0.12 and 0.17 (Table 4).

Variation in somatic mutation rates

Variation in proportions of *Onoclea sensibilis* plants segregating for mutations suggests variation in the underlying rate of somatic mutation among populations. If we assume that mutations arise during vegetative growth of the fern clone, it is possible to obtain an approximation of the amount of relative variation among populations in the rate of somatic mutation, i.e., variation in the rate of somatic mutation per unit of vegetative growth. This is the composite rate for all mutations that disrupt gametophyte development when passed on to the spore-producing portion of the plant (Klekowski and Klekowski 1982). Each sexual frond sampled is assumed to derive from a separate clone. The clones are assumed to have grown by the same
FIG. 5. Examples of Salmonella mutagenicity test results for organic extracts of soil samples: results from collection sites of two Onoclea sensibilis populations. Mutagenic potency (MP) values are the slopes of the initial linear portion of the concentration–response function. Potency unit is Salmonella revertents per milligram equivalent of dry soil.

amount, and somatic mutations are assumed to occur rarely. Under this set of assumptions, the proportion of mutation-free clones, \( P_0 = \frac{N_{\text{nonseg}}}{N_{\text{tot}}} \), should follow a Poisson distribution where the expected value of \( P_0 = e^{-u} \), \( u \) is the number of somatic mutations per unit of vegetative growth, and \( v \) is the number of units of vegetative growth. The maximum-likelihood estimate of \( u \) is \( \ln\left( \frac{N_{\text{nonseg}}}{N_{\text{tot}}} \right) \) (Koziol 1991). As \( v \) is unknown, we cannot obtain absolute estimates of the somatic mutation rates. The relative variation in \( u \) among populations, however, can be calculated from the data in Table 4 by noting that \( P_0 = 1 - p \). Using this approach, the rate of somatic mutation is estimated to be approximately 10-fold higher for some of the downstream vs. other populations (Table 4).

Ames tests of soil extracts and correlation with fern assay results

A pilot experiment conducted using the Sorel soil extract revealed a positive mutagenic response in the presence of S9 metabolic activation. Comparisons between the responses obtained using the metabolically enhanced strains YG1041 and YG1042 (mutagenic potential = 0.75 ± 0.22 and 1.46 ± 0.34 revertents per milligram equivalent of dry soil, respectively) and their parent strains TA98 and TA100 (mutagenic potential = 0.54 ± 0.11 and 1.08 ± 0.10 revertents per milligram equivalent of dry soil, respectively) revealed no significant increase in the mutagenic response in the presence of OAT and NR. It should be noted, however, that the results did reveal a slight, but significant, response on YG1041 without S9 activation. Concentration–response plots for 11 soil extracts were plotted, and the mutagenic responses assessed. Fig. 5 shows examples of TA98 and TA100 concentration–response plots for soil extracts from Contrecoeur and Lavaltrie.

Soil from the Sorel site elicited the greatest mutagenic response, while that from Baie-du-Febvre elicited the lowest. Six of the seven downstream samples examined (Sorel, Contrecoeur, Berthierville, Lanoraie, Lavaltrie, Repentigny) yielded significant positive responses. The remaining downstream sample, collected at Baie-du-Febvre, yielded a marginal response. Two of the three extracts from soil samples collected from upstream sites (Dollard des Ormeaux, Saint Anicet) yielded significant positive responses. The remaining upstream sample, collected at Senneville, yielded a marginal response. Two of the three extracts from soil samples collected from upstream sites (Dollard des Ormeaux, Saint Anicet) yielded significant positive responses. The remaining upstream sample, collected at Senneville, yielded a marginal response. Two of the three extracts from soil samples collected from upstream sites (Dollard des Ormeaux, Saint Anicet) yielded significant positive responses. The remaining upstream sample, collected at Senneville, yielded a marginal response. Two of the three extracts from soil samples collected from upstream sites (Dollard des Ormeaux, Saint Anicet) yielded significant positive responses. The remaining upstream sample, collected at Senneville, yielded a marginal response.

Mutagenic potency values, calculated for all extracts that yielded a well-behaved concentration–response function, are summarized in Table 5. The results reveal that the mutagenic potency was greater on the base-pair mutation strain TA100 (geometric mean = 0.48 revertents per equivalent dry milligram of soil) than on the frame-shift strain TA98 (geometric mean = 0.099 revertents per equivalent dry milligram of soil).
per milligram equivalent of dry soil). In addition, the mutagenic potency of extracts from downstream samples (geometric mean = 0.68 for TA100 and 0.12 for TA98) is greater than that of samples collected upstream of Montreal or away from the St. Lawrence River (geometric mean = 0.28 for TA100 and 0.062 for TA98). Statistical analyses, however, did not reveal significant differences between mean downstream mutagenicity and mean upstream/away mutagenicity at \( P < 0.05 \). Results from the two strains were significantly rank correlated (Spearman rank correlation = 0.83, \( P < 0.05 \)). Fig. 6 illustrates the relationship between the estimated proportion of ramets segregating for mutations (\( p \)) and the Salmonella mutagenicity of extracts from the surrounding soil. Additional analyses revealed a significant rank correlation between the estimated frequencies of \( O. sensibilis \) ramets segregating for mutation and the frameshift (i.e., TA98) mutagenicity of organic substances in the surrounding soil (Spearman rank correlation = 0.65, \( P < 0.05 \)). Although Fig. 6 also suggests a significant relationship between the estimated frequencies of \( O. sensibilis \) ramets segregating for mutation and the base-pair (i.e., TA100) mutagenicity of extracts from surrounding soil across several sites, statistical analyses did not reveal a significant rank correlation (Spearman rank correlation = 0.43, \( P = 0.17 \)).

**DISCUSSION**

**Regional variation in mutation rates and correlation between the fern and microbial assays**

The development of simple, short-term mutagenicity assays in the early 1970s led to the identification of a wide range of mutagenic substances (e.g., see McCann et al. 1975). Shortly after the description of the Salmonella mutagenicity assay, researchers began using the assay to investigate the presence of mutagenic substances in complex environmental samples (see Waters et al. 1979, 1981, 1983, and 1986 for a thorough overview). Although these research efforts successfully identified the mutagenic hazards of industrial wastes and effluents, contaminated soils, contaminated surface waters, combustion emissions such as cigarette smoke and vehicle exhaust, and chlorinated drinking water, an assessment of the effects manifested in situ has proved exceptionally difficult. A wide range of biomarkers has been used to document the exposure and effects of environmental mutagens (e.g., detection of contaminated tissues, fluorescent metabolites in bile, DNA adducts, and cytogenetic abnormalities), and many researchers have predicted dire consequences for individuals and populations (e.g., Kurelec 1993, Anderson and Wild 1994, Bickham and Smolen 1994). Although exposure and subsequent DNA damage are required for the formation of mutations, direct measurements of somatic or germinal mutation frequency in wild organisms has proved difficult and, for the most part, elusive. This study employed an in situ mutation assay system originally pioneered by Klekowski and colleagues to demonstrate that there are large proportions of plants segregating for mutations that disrupt gametophyte development in some populations of \( O. sensibilis \) and that there is regional variation among populations in the frequency of plants producing aberrant gametophytes. Most of the regional variation is attributable...
to differences between populations located downstream vs. upstream from the Montreal urban community or away from the St. Lawrence River. Spores obtained from plants in downstream sites yielded the highest estimated proportions of parents segregating for mutations. Moreover, the Salmonella mutagenicity assay revealed that organic extracts from soils collected at downstream sites have a higher mutagenic potency than those from soils collected at upstream sites or sites away from the St. Lawrence River. This result suggests that the mutagenic hazard identified via the Salmonella assay is being manifested in a local population of wild biota and that this short-term bioassay can provide information that is useful in evaluating the risk of genotoxic pollution to members of the native flora.

It should be noted that the relationship between the proportion of ramets segregating for mutation in *Onoclea sensibilis* populations and the Salmonella mutagenicity of soil extracts (Fig. 6) is empirical. Therefore, we cannot presume that the putative mutagens responsible for the soil mutagenicity results are also responsible for the mutagenic effects on *Onoclea sensibilis*. Comparisons between mutagenic effects in plants and mutagenic effects detected using the Salmonella/mammalian microsome system are complicated by dramatic physiological differences between bacteria (supplemented in vitro with mammalian microsomal enzymes) and plants. Nonetheless, several researchers have highlighted the ability of plants and green algae to activate promutagenic substances including polycyclic aromatic hydrocarbons and aromatic amines (Harwood et al. 1989, Plewa and Wagner 1993).

It should be noted that the Salmonella mutagenicity assay only measures sequence alterations such as frame shifts, transitions, and transversions. The assay cannot detect chromosomal abnormalities that may be partly responsible for the effects on *Onoclea sensibilis*. In a recent study of PAH-contaminated soils in the Czech republic, Chroust et al. (1997) noted that the frequency of heritable (gametic) mutations in *Arabidopsis thaliana* is highly correlated with the frequency of chromosomal abnormalities in *Vicia faba*. It would prove interesting to conduct additional investigations using assays capable of detecting chromosomal aberrations and/or the Salmonella mutagenicity assay with a metabolic activation mixture derived from the flora under investigation (i.e., *Onoclea sensibilis*).

Langevin et al. (1992) and White et al. (1998a, b) suggest that the St. Lawrence system is contaminated by a wide range of genotoxic organics. Although it is not our intention here to determine which compounds might be responsible for the mutation rate variation observed here, detailed examination of the soil data can provide a great deal of insight. Although the diagnostically strains YG1041 and YG1042 indicate that it is unlikely that the putative mutagens include aromatic amines, the weak positive response on YG1041 without S9 for the Sorel sample (not shown) does implicate nitroarenes, compounds frequently associated with diesel emissions (Sato et al. 1985, Tokiwa and Ohnishi 1986) and munitions wastes (Berthe-Corti et al. 1998). However, increases in the potency of nitroarenes and aromatic amines on OAT- and NR-enhanced strains are often one to three orders of magnitude above that observed with the parent strains (Hagiwara et al. 1993).

The lack of substantial enhancement in OAT- and NR-enhanced strains in the presence of S9 is in stark contrast to recent examinations of municipal waste-water and contaminated surface waters (White et al. 1999, Ohe et al. 2000). Analyses of municipal wastewater and surface water collected in the Providence River system (Rhode Island, USA) suggested that much of the mutagenicity could be accounted for by aromatic amines. Moreover, recent soil analyses have revealed
enhanced responses in OAT-enhanced Salmonella strains also (Watanabe et al. 1998). However, the results obtained in this study point towards unsubstituted, homocyclic PAHs as the putative mutagens. These substances always require S9 metabolic activation (or similar activation) and their base-pair activity is often greater than or equal to their frameshift activity (Sakai et al. 1985, DeMarini et al. 1994). In addition, several studies have revealed potent S9-activated mutagenicity in soils and sediments known to be contaminated with homocyclic PAHs such as benzo[a]pyrene, chrysene, benzo[a]anthracene, and benzo(b)fluoranthene (e.g., Metcalfe et al. 1990, Marvin et al. 1993, Donnelly et al. 1995, LaRocca et al. 1996). Additional analyses by Marvin et al. (1993) also revealed that sediment extract mutagenicity for samples contaminated with homocyclic PAHs is not enhanced on OAT- and NR-enhanced strains. Although some industrial and municipal wastewaters do contain mutagenic PAHs (White et al. 1996, White and Rasmussen 1998), PAHs in the upper horizons of urban/residential soils are generally thought to be PAHs associated with airborne particulate material that enter soil via dry deposition (Görgelmann and Spitzauer 1983, Jones et al. 1989, Fromme et al. 1993, Pathirana et al. 1994, Nielsen et al. 1996).

It is interesting to compare the soil results obtained in this study with a recent review of published soil mutagenicity data for 185 industrial, urban/residential, and rural/agricultural sites (P. A. White and L. Claxton, unpublished data). Several published studies describe TA100 mutagenicity of extracts containing homocyclic PAHs at frequencies greater than corresponding TA98 values (e.g., Metcalfe et al. 1990, Marvin et al. 1993, LaRocca et al. 1996). Additional analyses by Marvin et al. (1993) also revealed that sediment extract mutagenicity for samples contaminated with homocyclic PAHs is not enhanced on OAT- and NR-enhanced strains. Although some industrial and municipal wastewaters do contain mutagenic PAHs (White et al. 1996, White and Rasmussen 1998), PAHs in the upper horizons of urban/residential soils are generally thought to be PAHs associated with airborne particulate material that enter soil via dry deposition (Görgelmann and Spitzauer 1983, Jones et al. 1989, Fromme et al. 1993, Pathirana et al. 1994, Nielsen et al. 1996).

It is important to consider the consequences of elevated deleterious mutation rates for the long-term viability of the affected populations. Somatic mutations in plants are passed on to gametes and gametophytes, and therefore, they can contribute to a corresponding increase in genomic mutation rates. Important and unanswered questions are whether loci that affect sporophyte fitness are also mutating at higher rates in the impacted populations, and, if they are, what their dominance coefficients and selective effects are. Clearly, lethal mutations expressed in gametophytes, as assayed in this work, will not be passed on to future generations. Their main fitness consequence is in reducing the reproductive output of the parents in the current generation. If we assume, however, that sporophytically expressed loci also exhibit elevated rates of mutation, and that many of these mutations are of mild effect (and thus resistant to selective purging), we can appeal to the theoretical analysis of Lynch et al. (1995a, b) to examine the potential population viability consequences of our findings. Briefly, Lynch and his colleagues postulate a connection between a population’s mutational load and its rate of growth. They show that populations of small to moderate effective size (N e ≲ 1000) are likely to accumulate mildly deleterious mutations by genetic drift. Under the hypothesis that increasing mutation load reduces population growth rate, population growth will decline and eventually fall below replacement level, whereupon the population enters a positive feedback phase in which declining numbers lead to accelerating mutation accumulation (the “melt-
down” phase), followed rapidly by population extinction.

Lynch et al. (1995a, b) have shown that the expected time to extinction is a function of the spontaneous mutation rate for all genes influencing fitness, distribution of effects of mutations, average degree of dominance of mutations, population growth rate, and population carrying capacity. For deleterious mutations of equal effect that interact additively, changes in the mean time to extinction of a population are roughly inversely proportional to changes in the deleterious mutation rate. According to the model results, a doubling of mutation rate is expected to reduce population persistence time by 50%. For the fern populations we studied, if the 5- to 10-fold increase in mutation rate observed with gametophytic lethals is characteristic of the overall genomic mutation rate of these populations, this could lead to significantly reduced population persistence times.

Future work

Several results obtained here would benefit from further study. It would be useful to investigate whether soil contaminants could have led to epigenetic modification of the physiology of Onoclea spores. Despite the observed bimodal distribution of proportions of normal gametophytes produced by the fern clones (Fig. 4), a result that suggests a genetic basis for the underlying results, it cannot be entirely ruled out that some of the aberrant gametophytes arose due to direct effects of soil contamination on the sporophyte (i.e., rather than through mutations passed on to the spores and gametophytes). It is known that conditions under which spores are stored and mature may alter spore germination (Raghavan 1989).

We also suggest that it is important to determine whether other species in this community have been similarly affected by environmental contaminants. Unfortunately, with the exception of ferns like Onoclea sensibilis, few organisms easily lend themselves to this type of analysis. One possible strategy is to use single- or multilocus DNA fingerprinting to assess the rate of heritable mutation in naturally occurring plants or animals for which parents and progeny can be readily identified. This technique was employed by Yauk and Quinn (1996) to demonstrate an elevated rate of heritable mutations in Herring Gulls nesting at urban/industrial sites. In addition, transgenic plants such as the Arabidopsis thaliana employed by Kovalchuk et al. (1998) to assess the chronic hazards of radioactive contamination near the Chernobyl nuclear power facility could be used to measure the frequency of homologous recombination in plants exposed to soils collected in natural habitats. Subsequent analyses could also quantify the concentration of specific mutagenic PAHs and employ the ratio of alkylated PAHs to nonalkylated PAHs in order to verify whether the PAHs in the contaminated soils are actually pyrogenic in origin (LaRocca et al. 1996, Boehm et al. 1998).

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