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Population Biology

Allozyme and restriction fragment length polymorphism analyses confirm *Entomophaga maimaiga* responsible for 1989 epizootics in North American gypsy moth populations

(insect pathology/fungal pathogen/epizootiology/biological control)

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ABSTRACT **In 1989, populations of North American gypsy moth, *Lymantria dispar*, in seven contiguous northeastern states were severely reduced by a fungal pathogen. Based on morphology, development, and pathology, this organism appeared to be *Entomophaga maimaiga*. We have now used allozyme and restriction fragment length polymorphism analyses to confirm this identification. Previously, this mycopathogen had been reported only from gypsy moth populations in Japan. During 1989, *E. maimaiga* occurred only in areas that had been initially defoliated by gypsy moth >10 years ago. *E. maimaiga* caused 60–88% mortality in late instar larvae on research sites in central Massachusetts.**

The gypsy moth, *Lymantria dispar*, is capable of sustained outbreaks lasting from 1 to many years (1). Outbreaks of this important forest defoliator are frequently terminated by dramatic population declines due to a commonly occurring nucleopolyhedrosis virus [*L. dispar* nucleopolyhedrosis virus (LdMNPV)]; LdMNPV generally causes epizootics only in dense populations (1). In 1989, northeastern gypsy moth populations were starting to increase after 7–8 years at low densities. Extensive mortality occurring in 1989 North American gypsy moth populations was caused by a previously unreported pathogen in the fungal group Entomophthorales (2). The dramatic impact of this pathogen on less dense populations of gypsy moth necessitated identification of this fungus and documentation of its impact.

Azygospores and conidia typical of fungi in the *Entomophaga aulicae* species complex were produced by cadavers of gypsy moth larvae collected in 1989. The *E. aulicae* species complex occurs worldwide in the northern hemisphere and members infect hosts in nine families of Lepidoptera (refs. 3–5; R.A.H., unpublished data). This complex is at present poorly defined and only one species within it, the gypsy moth pathogen *Entomophaga maimaiga*, has been adequately described (6). Based on morphology, development, and pathology, the 1989 northeastern fungus appeared to be *E. maimaiga* (2). Fungal morphology alone cannot distinguish members of the *E. aulicae* complex and too little is known of development and pathology for these attributes to be used definitively for species identification. In the species description for *E. maimaiga*, the clearest criterion separating this species from other members of the complex is isozyme composition (6).

Restriction fragment length polymorphisms (RFLPs) have also been used successfully to differentiate fungal isolates within and between species (7–9). The existence of species-specific RFLP patterns has been demonstrated among 24 different *Entomophaga* isolates and a clear pattern has emerged differentiating isolates of *E. maimaiga* from a general *E. aulicae* pattern (S.R.A.W. and J.C.S., unpublished data). However, allozyme and RFLP analyses have never been used in concert to evaluate whether results are comparable.

In the present study, we document isolation of the 1989 fungal pathogen of gypsy moth and its identification using allozyme and RFLP assays. Both types of assays were considered necessary for conclusive identification due to the lack of definition in the *E. aulicae* species complex. The distribution of this pathogen in the northeast was intensively determined relative to the spread of gypsy moth. The quantitative impact of this pathogen on late larval stages of gypsy moth is presented and historical aspects of the impact of this pathogen are discussed.

MATERIALS AND METHODS

Pathogen Isolation. Cadavers and infected larvae collected in areas experiencing epizootics were used for fungal isolation. Conidia and conidiophores from cadavers, as well as hemolymph from living infected insects, were added to Grace's insect tissue culture medium (GIBCO) or M199 medium (GIBCO), both with and without 5.0% fetal bovine serum.

Identification of the Pathogen. Horizontal starch gel electrophoresis following the methods of May *et al.* (10) was used to compare the 3 northeastern isolates with (*i*) 6 Japanese isolates of *E. maimaiga* (4 isolates from the west coast and 2 isolates from the east coast of Honshu), (*ii*) 20 isolates of the *E. aulicae* species complex from 10 lepidopteran host species collected at 15 sites [Tortricidae: *Choristoneura fumiferana* (6 isolates, Maine; 2, Ontario, Canada), *Pseudaletia* sp. (1, Ontario); Geometridae: *Lambdina fiscellaria* (1, Newfoundland, Canada), *Lambdina fiscellaria lugubrosa* (1, British Columbia, Canada), *Enypia griseata* (1, British Columbia); Lasiocampidae: *Dendrolimus spectabilis* (1, Ibaraki Prefecture, Japan); Notodontidae: *Heterocampa guttivitta* (1, Vermont); Noctuidae: *Aedia leucomelas* (1, Fuchu City, Tokyo, Japan), *Heliothis* sp.? (1, Georgia), *Mamestra brassicae* (2 isolates Ibaraki Prefecture); unknown family and species: (2,

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Abbreviations: RFLP, restriction fragment length polymorphism; LdMNPV, *Lymantria dispar* nucleopolyhedrosis virus; rDNA, rRNA-encoding DNA.

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Ibaraki Prefecture)], and (*iii*) 4 isolates of the closely related *Entomophaga grylli* species complex from 4 orthopteran hosts collected at 4 sites [Acrididae: *Parapodisma* sp. (1 isolate, Fukui Prefecture, Japan), *Melanoplus differentialis* (1, Kansas), *Camnula pellucida* (1, Arizona), *Aiolopus thalassinus tamulus* (1, Cikampek, Java)]. All 30 isolates were examined for genetic variability at nine allozyme-coding loci (*Lap*, leucine aminopeptidase; *Gr-1* and *Gr-2*, glutathione reductase; *Gpi*, glucosephosphate isomerase; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase; *Pgk*, phosphoglycerate kinase; *Mdh-2*, malate dehydrogenase; *Pgm*, phosphoglucomutase; *Pep-GL*, peptidase with glycylleucine).

Using an rRNA-encoding DNA (rDNA) probe from the yeast *Saccharomyces cerevisiae* (11), fungal isolates were tested for polymorphisms in nuclear rDNA sequences for the restriction enzyme *Hin*dIII following the general methodology of Maniatis *et al.* (12). A second probe (SW16) consisting of a cloned fragment of *E. aulicae* DNA was also used. The three northeastern isolates were compared with an *E. aulicae* isolate from *Choristoneura fumiferana* collected in Newfoundland, Canada, that displays the typical *E. aulicae* pattern as well as an *E. maimaiga* isolate from gypsy moth collected on the western coast of Honshu, Japan, and an *E. grylli* isolate from *Dissosteira carolina* (Acrididae) collected in Ontario, Canada.

Epizootiological Studies. To determine the distribution of *E. maimaiga* epizootics, surveys of 144 sites in 10 states were conducted during late June and July 1989.

Gypsy moth density and mortality were monitored in four forest stands dominated by oak (*Quercus* spp.) near Quabbin Reservoir in central Massachusetts. Egg mass densities were determined in autumn 1988 and 1989 by making counts inside 15 circles (7.5 m radius) in each stand (13). Between 19 June and 13 July 1989, weekly counts of gypsy moth larvae were made under burlap bands (14) wrapped around the stems of *ca.* 320 trees per stand. Mortality from *E. maimaiga* during the late larval stage was estimated by making weekly collections of *ca.* 100 larvae per stand; these larvae were subsequently reared in individual containers on artificial diet (15). Larvae were checked twice a week; cadavers were examined microscopically for the presence of *E. maimaiga* spores or LdMNPV occlusion bodies. Total proportion dying from *E. maimaiga* (PEM) was estimated as follows: PEM = $1 - S_1 \cdot S_2 \cdot S_3 \cdot S_4$, where $S_i = 1 -$ proportion dying from *E. maimaiga* during week i and $i = 1, 2, 3$, and 4 for the 4-week period when late-instar larvae were present.

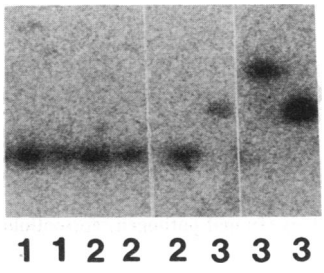

FIG. 1. Horizontal starch gel electrophoresis banding pattern for the glucosephosphate isomerase locus using buffer system 4 (10): Group 1, two isolates of *E. maimaiga* from Japan; group 2, isolates from infected *L. dispar* larvae from epizootics in Connecticut, Massachusetts, and New Hampshire; and group 3, three isolates from the *E. aulicae* species complex (left to right: *Heterocampa guttivitta*, Vermont; *Pseudaletia* sp., Ontario, Canada; *Dendrolimus spectabilis*, Ibaraki Prefecture, Japan).

RESULTS

Vegetative protoplasts of the northeastern fungus were isolated from the hemolymph of infected larvae collected in Connecticut, Massachusetts, and New Hampshire. They readily grew only in Grace's insect tissue culture medium supplemented with fetal bovine serum. Mycelia grew only on egg yolk/Sabouraud maltose agar (16). Isolates of *E. maimaiga* from Japan grow *in vitro* under the same specialized culture conditions (6). Northeastern isolates were also similar to *E. maimaiga* in dimensions and appearance of conidia, resting spores, and protoplasts (2, 6).

Based on allozyme analyses, banding patterns of the northeastern isolates were exactly the same as Japanese *E. maimaiga* for the nine loci examined (Fig. 1); no intraindividual variation within isolates was observed for any of these nine loci. The Japanese *E. maimaiga* and 1989 northeastern isolates differed from all *E. aulicae* isolates at five or six of the total nine loci examined (all except *Gr-2* and *Lap*). Results duplicated the previously published differences between

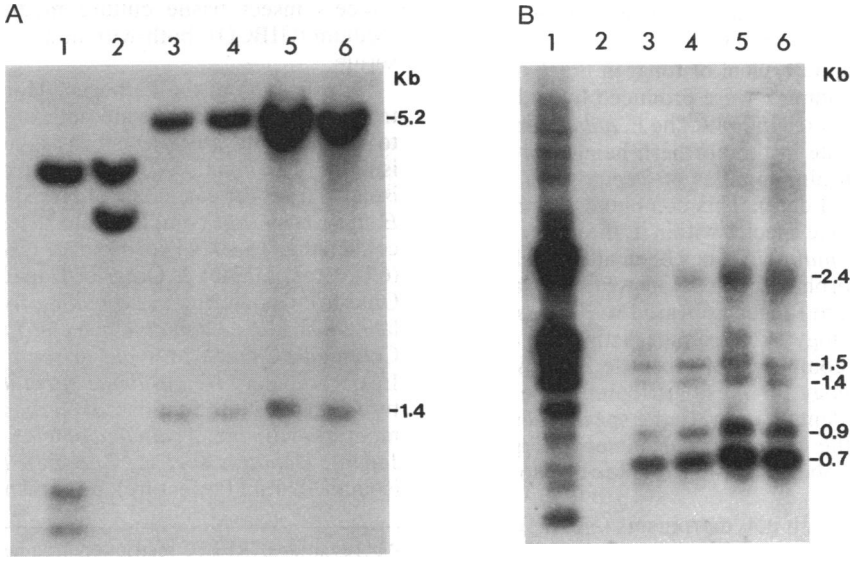

FIG. 2. (*A*) Southern hybridization of *Hin*dIII-digested *Entomophaga* DNAs with a radiolabeled *S. cerevisiae* rDNA probe. Various amounts of DNA were electrophoresed. Lane 1, 5 μg of *E. aulicae* DNA; lane 2, 5 μg of *E. grylli* DNA; lane 3, 4 μg of *E. maimaiga* DNA; lane 4, 5 μg of DNA from Connecticut isolate; lane 5, 6 μg of DNA from New Hampshire isolate; and lane 6, 6 μg of DNA from Massachusetts isolate. (*B*) Southern hybridization of same blot as in *A*, hybridized with the radiolabeled SW-16 probe. Kb, kilobases.

North American isolates from *C. fumiferana* and Japanese isolates of *E. maimaiga* at *Gpi*, *Gapdh*, *Pep-GL*, and *Gr-1* (6); in the present study, differences were also consistently found at *Pgm*, which was not tested previously. Variability in banding patterns among the 20 *E. aulicae* isolates tested confirmed that this group is a species complex. The Japanese *E. maimaiga* and 1989 northeastern isolates differed from *E. grylli* isolates at four to eight loci (all except *Lap*) of the total nine loci tested.

Each of the three northeastern isolates exhibited RFLP patterns with *Hin*dIII that differed from the patterns observed for *E. grylli* or *E. aulicae* but were identical to those observed for the Japanese *E. maimaiga* isolate (Fig. 2*A*). These results were confirmed using the second RFLP probe, SW16 (Fig. 2*B*). Thus, the allozyme and RFLP results support our view that these isolates from the northeastern panzootic cannot be distinguished from Japanese isolates of *E. maimaiga* and, therefore, are probably of recent common ancestry.

From survey samples, the presence of *E. maimaiga* was detected in seven contiguous northeastern states (Massachusetts, Connecticut, New Hampshire, Vermont, New York, Pennsylvania, and New Jersey) (Fig. 3). Based on the documented spread of defoliation by gypsy moth populations between 1910 and 1987 (Fig. 3), the current *E. maimaiga* panzootic occurred in many areas where gypsy moth populations caused defoliation in 1934 and 1957 but not in all areas defoliated in 1980. In addition, many of the areas that gypsy moth populations have colonized more recently and where *E. maimaiga* was not evident (e.g., central Pennsylvania and western New York state) received abundant rainfall during spring 1989 (17) (Fig. 3). *E. maimaiga* was not found in samples collected from the sites in southwestern New York and northwestern Virginia where a 1984 Japanese isolate of *E. maimaiga* was released in 1985 and 1986 (A.E.H. and R. S. Soper, unpublished data).

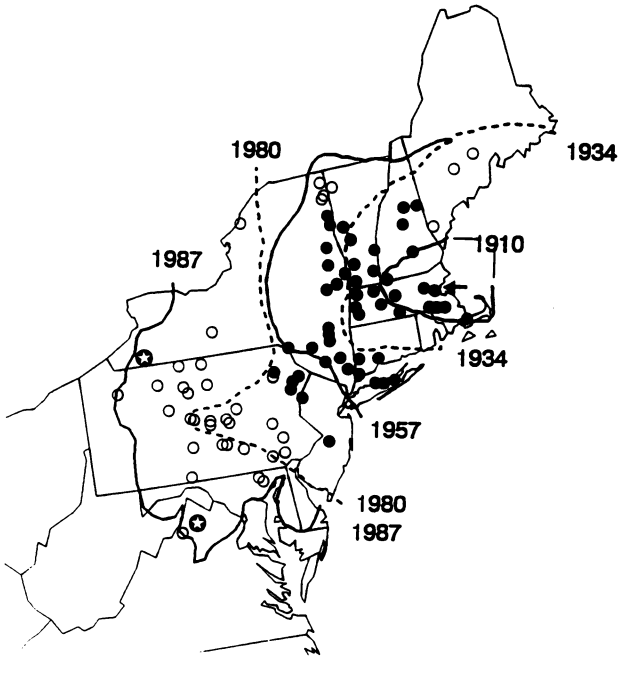

FIG. 3. Forest tree defoliation from spreading gypsy moth populations, 1910–1987 and 1989 surveys for *E. maimaiga* in the northeastern United States. Lines indicate limits of defoliation caused by gypsy moth spreading from its original introduction site near Boston (arrow). ●, Extent of the *E. maimaiga* epizootics during June and July 1989; ○, collection sites where *E. maimaiga* was not found in cadavers; ✪, points of 1985–1986 intentional introductions of 1984 isolate of *E. maimaiga*.

Table 1. Egg mass density of *L. dispar* populations and mortality caused by *E. maimaiga* on research sites in central Massachusetts, 1989

Stand	Density,* 1988 egg masses per hectare	% late instar mortality due to *E. maimaiga*	Density,* 1989 egg masses per hectare
A	26.4 ± 3.7	87.7	45.3 ± 15.3
B	11.3 ± 6.5	78.2	42.5 ± 11.6
C	26.4 ± 3.7	78.6	42.5 ± 9.7
D	15.1 ± 9.9	60.3	19.8 ± 7.1

One hectare = 10^4 m^2.
*Values are expressed as mean ± SEM.

In four research plots in central Massachusetts, mortality from *E. maimaiga* among late-instar larvae ranged from 60% to 88% (Table 1). Despite these high levels of mortality, there was relatively little change from 1988 to 1989 in gypsy moth egg mass densities, which remained well below outbreak levels. Because of the high fecundity of gypsy moths (*ca.* 600 eggs per mass in these populations), mortality in immature stages in excess of 80% may not be sufficient to cause population decline. The results suggest that without the mortality due to the *E. maimaiga* epizootic, a marked increase in gypsy moth density probably would have occurred.

DISCUSSION

This study documents use of allozyme and RFLP analyses in concert in insect pathology. The results of these sensitive tests corroborate each other and lend certainty to the identification of *E. maimaiga*. Similar analyses including as yet uncollected entomophthoralean pathogens of gypsy moth reported from China (18), Korea (R. W. Pemberton, personal communication), and Poland (19) may further substantiate that the 1989 northeastern *E. maimaiga* strain originated in Japan (3, 6).

A unique feature of this study is that mortality levels have been quantified; estimation of host mortality during fungal epizootics is frequently impossible. High levels of mortality due to *E. maimaiga* occurred in late instars even in the low level populations in central Massachusetts. In higher density populations in Connecticut, fungal infection levels were not quantified, but *E. maimaiga* was considered to have prevented extensive defoliation (2). The importance of *E. maimaiga* mortality in low as well as high density populations suggests that the activity of this pathogen may be less dependent on host density than the LdMNPV. Unfortunately, the overall impact of *E. maimaiga* on gypsy moth populations cannot be known because fungal infection levels in early instars during 1989 epizootics were not documented.

The widespread epizootics caused by a previously unreported fungus specific to gypsy moth raises questions regarding the origin of *E. maimaiga* in North America. Based on samples taken in New York and Virginia following 1985 and 1986 field releases of the 1984 Japanese isolate of *E. maimaiga*, this isolate of *E. maimaiga* caused only extremely low levels of infection. No evidence of *E. maimaiga* was found among cadavers collected from these release sites during 1989. Thus, it is highly unlikely that the 1989 epizootics were derived from these recent small-scale intentional introductions of *E. maimaiga*. Instead, the near-ubiquitous occurrence of *E. maimaiga* in regions long infested by gypsy moth (Fig. 3) suggests a much earlier introduction.

It is most likely that *E. maimaiga* may not have been detected in previous years because cadavers of larvae killed by *E. maimaiga* look very similar to cadavers of LdMNPV-killed larvae to untrained observers; the conidia and conid-

iophores present externally on cadavers are very short-lived and usually only microscopic examination confirms the presence of *E. maimaiga*. Also, *E. maimaiga* may not be common most years. High humidities are required for conidial production, and free water is necessary for conidial germination (20). Rainfall in May 1989 was the second highest on record since 1931 in Connecticut and western Massachusetts, and June rainfall was well above average (17). Furthermore, gypsy moths were relatively abundant in 1989 compared with many years during which densities are so low that larvae are almost never observed and mortality would go unnoticed.

Previous analyses of long-term data on gypsy moth egg mass density collected between 1911 and 1932 at sites throughout the northeastern United States indicated that June rainfall was strongly associated with population decline (1, 21). In particular, a collapse of high density populations in 1922 was associated with heavy June rainfall. Such population declines have usually been attributed to epizootics of LdMNPV. Other studies conducted in 1958–1963 revealed that disease incidence was higher on wetter sites (1, 21), leading to the belief that wet years promote epizootics of LdMNPV. Preliminary laboratory studies (22) indicated a positive relationship between relative humidity and incidence of LdMNPV; however, these findings have not been subsequently corroborated (23). Late instar gypsy moth larvae acquire lethal infections of LdMNPV primarily by ingesting foliage contaminated with LdMNPV occlusion bodies deposited by dying larvae (24, 25). Rain might promote LdMNPV infections by dispersing LdMNPV more evenly across the habitat, but rain is also known to wash such particles from the foliage (thereby reducing inoculum available to gypsy moth larvae). Given the similar appearance of larvae killed by LdMNPV and *E. maimaiga*, we suspect that many previous collapses of high density gypsy moth populations in North America correlated with wet conditions may in fact have been caused by *E. maimaiga*.

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