F-1.2.1 Effects of disease on individual fitness components and population viability

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Abstract Ecological and genetic interactions between *Eupatorium makinoi* and geminiviruses were examined. Virus infection decreased growth rate and survivorship under controlled condition and was a major mortality factor in the field. While geminiviruses were genetically highly variable in the host-range gene, suggesting high ability of adaptive changes against host's resistance, *E. makinoi* had genetically variable resistance genes. We investigated prevalence of canine distemper virus (CDV) infection in Japanese dogs genetically, seroepidemiologically and histopathologically. Japanese racoon dogs were naturally infected with recent type of CDV which are prevalent in dogs. Aquatic mammals on Japanese coast, in Caspian Sea and in Lake Baikal were also naturally infected with morbillivirus in recent years.

Key Words viral disease, resistance, genetic variation, canine distemper virus, morbillivirus

1. Ecological and genetic interactions between *Eupatorium makinoi* and geminiviruses

1.1. Research objectives

It is often claimed that epidemic disease is one of the major threats for persistence of wild populations. However, only few studies have been made on plant population dynamics under epidemics in the field. It is also uncertain whether or not populations having low genetic variation are more vulnerable to epidemic disease. This study was undertaken to describe population dynamics of *Eupatorium makinoi* under epidemics of viral disease, and to examine the process of antagonistic coevolution between the host plants and geminiviruses.

1.2. Materials and methods

Eupatorium makinoi is a perennial plant, consisting of sexual and agamospermous populations. Agamospermous populations are more abundant and widespread. Agamospermous plants are more fertile than sexual plants and colonise disturbed habitats frequently and establish a large population rapidly because they produce abundant seeds without fertilisation. However, agamospermous populations often suffer high incidence of geminivirus infection. Infected plants are easily identified by the visible symptom of yellowed veins. We followed dynamics of three populations in the field under epidemics.

Also, performance of infected and non-infected plants was compared in the greenhouse under controlled light environment.

Among plant viruses, most of which have RNA genomes, geminiviruses are characterised by having DNA genomes. Because it can be utilised as a vector for genetic engineering in plants, it has been the focus of intensive molecular biological studies during the last decades. As a result, genome structure, gene arrangement and the function of each gene are now well understood. The genome of geminiviruses is uni- or bipartite. Bipartite viruses have DNA genomes approximately 2700 nucleotides of each. The host range of geminiviruses is rather narrow, and some genes are known to function in host range determination. In unipartite geminivirus, a small ORF C4 has been shown to encode a host range gene. In this study, total DNA was isolated from infected leaves of E. makinoi and DNA fragments containing ORF C4 were amplified with a set of specific primers. Sequences determined were subjected to molecular phylogenetic analyses using maximum parsimony or NJ method.

To examine genetic variation of resistance genes in E. makinoi populations, we amplified DNA fragments of E. makinoi using primers designed based on conservative motifs of previously known sequences of NBS-LRR type resistance genes.

1.3. Results and discussion

1.3.1. Effects of virus infection in the field

We studied the effects of virus infection on dynamics of *E. makinoi* populations in contrasting light environments, Gora-dani (a shaded population), and Minou-1 and Minou-2 (open-site populations)[1]. Censuses of the plants were taken for 8 years in Gora-dani and 4 years in Minou-1 and Minou-2. After the epidemics of virus infection, most plants were infected by virus in both sites. The plant number and the proportion of flowering plants decreased rapidly and simultaneously in the shaded population in Gora-dani. On the other hand, in the open-site populations of Minou, the proportion of flowering plants decreased first and then the number of plants decreased gradually. Growth analysis of the plants in the Gora-dani population revealed that height growth was significantly suppressed by infection, and that occurrence of flowering and survivorship of the infected plants were negatively correlated with plant height. Since the light availability affected plant growth and thereby flowering and survivorship, the difference in population dynamics between the two field sites would be caused by the differences in light environments. Although populations in open sites may persist for considerable periods after the virus epidemics, the individual local populations of *E. makinoi* would eventually go extinction irrespective of light environments.

1.3.2. Effects of virus infection in controlled light environments

We examined the effects of geminivirus infection on fitness components and on photosynthetic properties of the host plant, *E. makinoi*, grown at two irradiance levels in a natural-light greenhouse[2]. Under the low light condition (13% full sunlight), more than a half of the infected plants died during the 9-month experiment, while most of uninfected plants survived. Growth rate was also lowered by infection. At high light (50% full sunlight), by contrast, virus infection did not cause mortality despite slight decrease in growth rate. Flowering occurred only at high light, and reproductive outputs of the plants were markedly reduced by the infection. Infected leaves had distinct yellow variegations and, when compared with uninfected leaves, they showed (1) comparable light-saturated photosynthetic rate per unit area, but (2) lower initial slope of light-response curve of

photosynthesis on an incident irradiance basis. The lower initial slope was mainly due to reduction of light-harvesting chlorophyll-protein complexes in the variegated parts. Since the differences in plant performance, depending both on infection and on growth irradiance, were largely explained by the differences in growth rate and/or plant size, the reduced photosynthetic production in the infected plants would be a major factor explaining the inferior performance of the host plants.

1.3.3. Molecular divergence of geminiviruses

Few studies have been made on the molecular divergence of plant viruses. To remedy this deficiency, we examined the molecular divergence of the tobacco leaf curl geminivirus (TLCV)[3]. TLCV infects not only tobacco but also *Eupatorium* and *Lonicera* in the field and causes yellow vein disease. A total of 29 nucleotide sequences of the replication protein gene (ORF C1) of geminiviruses infecting wild plants of *E. makinoi*, *E. glehni* and *L. japonica* collected from ten localities was determined, Highly divergent sequences were obtained not only among host plant populations but also within a host population. Phylogenetic analyses showed that the TLCVs infecting *Eupatorium* and *Lonicera* were clustered into three different clades, and were either paraphyletic or polyphyletic, This result is the first evidence demonstrating that wild populations of single plant species possess genetically diversified virus strains. Comparison with recently reported genetic variations of tobacco mild green mosaic tobamovirus (TMGMV) revealed three characteristics of TLCV evolution: (1) a higher nucleotide substitution rate, (2) more frequent migration among geographically isolated host populations, and (3) more frequent host changes to different plant families. While TMGMV is an RNA virus, TLCV has DNA genomes. In animal viruses, RNA viruses tend to evolve faster than DNA viruses. Our results indicated that this trend might not hold for plant viruses.

1.3.4. Molecular adaptation of geminiviruses

One of the most promising hypotheses for the evolution of sex is that sexual reproduction is advantageous because it increases the rate of adaptive evolution in response to parasites[4]. To investigate this advantage of sex, we compared genetic variation of geminiviruses infecting sexual and asexual populations of *Eupatorium* (Asteraceae). The infection frequency was 37.5% in the sexual population and 87.8% in the asexual population. The lower infection frequency in the sexual population might be the result of higher genetic diversity of host plants. If geminiviruses have diverged to counter defence systems of genetically variable hosts, genetic diversity of viruses is expected to be higher in sexual host populations than in asexual host populations. To test this expectation, we used single-strand conformation polymorphism (SSCP) analysis to examine genetic diversity of the geminiviruses in a DNA region containing the open-reading frame (ORF) C4 gene, which is known to function as a host range determinant. As predicted, higher genetic diversity of viruses was observed in the sexual population: three SSCP types were found in the asexual population while six types were found in the sexual population. Sequencing of the polymerase chain reaction (PCR) products revealed further genetic diversity. Phylogenetic analysis of the sequences showed that the SSCP types belonged to four different clades. Several SSCP types from the same clade were found in the sexual population, whereas the asexual population included only one SSCP type from each clade. Amino acid replacements of ORF C4 are suggested to be accelerated in the sexual population. This evidence supports the hypothesis that sexual reproduction is advantageous as a defence against epidemic disease.

1.3.5. Divergence of NBS-LRR resistance gene homologues

To elucidate whether *E. makinoi*_has resistance genes with specificity against various pathogens, we amplified DNA fragments of the plants using a set of primers (Sense: 5'-GGIGGIRTIGGIAAIACIAC-3', ANTISENSE: 5'-IAGIGYIAAIGGIAGICC-3'), that were designed based on conservative motifs in NBS-LRR type resistance genes previously sequenced from various crops. Agarose gel electrophoresis of PCR products derived from single host individual revealed a lot of fragments with variable size. These products were cloned and selected clones of 213-762bp were sequenced. By excluding pseudogenes having stopped codons within the sequence, we got 12 functional sequences that show homology with previously known sequences of NBS-LRR type resistance genes. Among them, 11 sequences were different at least in one amino acid, and one sequence was highly diverged from all other sequences. This finding shows that individual plants of *E. makinoi* have a family of well-diverged resistance genes. Molecular phylogenetic analyses of these sequences as well as previously reported ones showed that the homologues of *Eupatorium* were monophyletic.

1.3.6. Implications on biological conservation

The results described above clearly showed that virus epidemics did cause local extinction of wild populations. Thus, control of epidemic disease is of primary importance in biological conservation. The results of this study also provided evidence of antagonistic coevolution between the viruses and the host plants in the level of interacting genes. Thus, genetic variations of resistant genes are considered to be counter adaptation of host plants against pathogens. In this study, homologues of NBS-LRR type resistance genes were successfully sequenced from wild plants of Eupatorium and even single individual had diverged resistance genes. The method described in this study will be widely applied to wild plant populations in which conservation is needed.

Literature cited

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2. Prevalence of canine distemper virus infection in Japanese dogs and wild animals

2.1.Epidemiological observations on recent outbreaks of canine distemper in Tokyo area

Recent outbreaks of canine distemper virus (CDV) infection in Tokyo area were investigated on the basis of clinical features and serological test. The affected dogs were clinically classified into two groups; dogs with respiratory and gastrointestinal signs associated with central nervous system (CNS) signs, and those with CNS signs alone. Of 62

dogs examined, 34 belonged to the former and 28 to the latter. In immunoperoxidase assay, anti-Onderstepoort strain of CDV serum reacted at a low level against 2 field isolates of CDV. These results suggested the presence of different types of CDV population in the field.

2.2. Serological analysis of canine distemper virus using an immunocapture ELISA.

As a rapid sensitive method to detect CDV, an immunocapture enzyme-linked immunosorbent assay (ELISA) was performed. The sensitivity and specificity of the immunocapture ELISA were considered to be high enough. Virus neutralizing (VN) test was also established using the immunocapture ELISA. By using this test, the different cross VN titers between sera of dogs experimentally infected with the Onderstepoort strain and those with a field isolate of CDV were observed.

2.3. Expression of the nucleocapsid protein gene of the canine distemper virus

We constructed a cDNA clone of CDV encoding an entire nucleoprotein (NP) gene, by means of the reverse transcription-polymerase chain reaction. The cloned NP gene was inserted into the eukaryotic expression vector, pRVSV. After transfection of the plasmid into Vero cells, we examined the expression of CDV-specific NP antigen by means of indirect immunofluorescence assay (IFA) and western blotting, using various antibodies against NP of CDV and an antiserum against NP of measles virus. The CDV-NP specific antigen was detected in the nuclei of the cells transfceted with pRV-ON, by means of IFA with antibodies specific to the NP.

2.4. Histopathological features of canine distemper recently observed in Japan

Eleven dogs with canine distemper (CD) from the Chubu region of Japan and the Tokyo area were examined. Clinically, respiratory and neurological signs were present in all animals. Histopathologically, all showed characteristic CD lesions of bronchopneumonia and demyelinating encephalitis. However, some differences in gastrointestinal abnormalities were observed. Three out of four dogs from the Chubu region had severe diarrhea and gastroenteritis, associated with numerous eosinophilic inclusion bodies in the mucosal epithelia. The remaining dog from this area showed vomiting, but not diarrhea, and also had a number of intraepithelial inclusion bodies in the gastric and intestinal mucosa. In contrast, the seven dogs from the Tokyo area showed neither gastrointestinal symptoms nor intraepithelial inclusions in the stomach or intestine. Immunohistochemical examination for CDV antigens, however, revealed that these seven dogs had immunoreactive products in the mucosal epithelia, suggesting that the epithelial cells had either a low level of infection with CDV or were infected with a less cytopathogenic virus. These findings suggest that the dogs in this study were probably affected by two distinct types of CD, in terms of epitheliotropism and cytopathogenic effects on the gastrointestinal tissues.

2.5. Molecular and phylogenetic analyses of the haemagglutinin (H) proteins of field isolates of canine distemper virus from naturally infected dogs.

We isolated three strains of CDV from dogs in Japan and analysed the molecular properties of their haemagglutinin (H) proteins. Immunoprecipitation of all three strains with a

monoclonal antibody revealed H proteins with molecular masses of 84 kDa, which differs from the Onderstepoort vaccine strain. However, after tunicamycin treatment immunoprecipitation identified H proteins of identical molecular mass (68 kDa) for all three field isolates and the vaccine strain. Sequence analysis showed nine potential sites for asparagine-linked glycosylation in the H proteins of the new isolates, in contrast to four in the H protein of the Onderstepoort strain. Thus, variation in glycosylation of the H proteins of the isolates and the vaccine strain may cause differences in antigenicity of the viruses. Sequences of the H genes showed that the new Japanese isolates have 99% identity with each other, 95% with other European and American isolates (from seals, a German dog, a ferret and large felids) and 90% with the vaccine strain. Phylogenetically, the new Japanese isolates form one cluster which is separate from recent European or American isolates, all of which are distinct from vaccine strains.

2.6. The nucleotide and predicted amino acid sequence of the fusion protein of recent isolates of canine distemper virus in Japan.

Analysis of the molecular properties of fusion (F) proteins of field isolates of CDV by immunoprecipitation analysis revealed an identical molecular mass of F protein of 3 field isolates with the Onderstepoort strain at nucleotide and amino acid levels, respectively. All of the 13 cysteine residues and 4 potential asparagine-linked glycosylation sites were completely conserved amongst these strains. These results indicate that the F protein is much less heterogeneous than that observed in the H protein of CDV.

2.7. Molecular identification of a recent type of canine distemper virus in Japan by restriction fragment length polymorphism.

Restriction fragment length polymorphism analysis was used to differentiate recent field viruses of CDV from vaccine strains. Virus genomes were amplified by using reverse transcriptase-polymerase chain reaction in part of the haemagglutinin gene. After digestion with EcoRV, the PCR products of recent field isolates were cut into two fragments that differ from the uncut form of old strains including all of vaccine strains. This method could be applied to fresh or stored brains, spleens and peripheral blood mononuclear cells of infected dogs. This molecular approach is useful for determining the causative agent of post-vaccinated CDV infection.

2.8. A canine distemper virus epidemic in racoon dogs (*Nyctereutes procyonoides viverrinus*) in Japan.

Antibody against canine distemper virus (CDV) was detected by ELISA and by a virus-neutralising test in blood samples from 75 free-ranging racoon dogs between 1982 and 1998 in Japan. We isolated CDV from an affected racoon dog. Molecular analysis revealed that this virus belonged to the same branch as recent Japanese isolates from field dogs on the phylogenetic tree of the H gene. The apparent molecular size of the H protein of the virus in immunoprecipitation was the same as that of the recent isolates from dogs and larger than that of a vaccine strain. One antigenic difference defined by a monoclonal antibody and only four differences of the deduced amino acid sequence of the H gene were found between the racoon dog virus and recent dog isolates. Thus, the virus spreading in Japanese racoon dogs is in the same group as the CDV strains causing recent outbreaks in dogs but with some differences.

2.9. Seroepidemiological survey of morbillivirus infection in aquatic mammals on the coast of Japan.

We conducted serological survey on morbillivirus infections in aquatic mammals on the coast of Japan. Sera were analysed by VN test against CDV and ELISA against CDV or phocine distemper virus (PDV). The prevalence of anti-CDV antibody and mean of the VN titers in the cetaceans were 0.0% to 1993, 50.0% and 45.0 in 1996 and 15.7% and 20.1 in 1997. All of the positive sera were collected from cetaceans captured in the Pacific Ocean. The percentage of the pinnipeds whose sera reacted with PDV were 100% in 1994, 20.0% in 1995, 71.4% in 1996, 45.0% in 1997 and 62.6% in 1998. The results indicate the presence of morbillivirus infections in cetaceans before 1996 and in pinnipeds before 1994 on the coast of Japan.

2.10. Seroepidemiological survey of morbillivirus infection in seals in Caspian Sea and in Lake Baikal.

Total of 24 sera of Caspian seals and 7 sera of Baikal seals were examined by VN test using CDV and ELISA against CDV and PDV. In Caspian seals, 1 of 14 in 1993 and 9 of 10 in 1997 were seropositive by VN test, and the titers were high in 1997. Of 7 sera of Baikal seals collected in 1998, 6 samples showed positive both VN test and ELISA. These results suggest that morbillivirus epizootic in Caspian seals caused in 1996 or early 1997 and CDV infection had been maintained in Baikal seals in recent years.