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Cutaneous and Systemic Poxviral Disease in Red (*Tamiasciurus hudsonicus*) and Gray (*Sciurus carolinensis*) Squirrels

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Abstract. From September 2005 through October 2006, fibromatosis was diagnosed in 2 red squirrels (*Tamiasciurus hudsonicus*) and 1 gray squirrel (*Sciurus carolinensis*). All 3 squirrels had multifocal to coalescing, tan, firm alopecic cutaneous nodules. Two squirrels also had pulmonary nodules. Histologically, the cutaneous nodules had marked epidermal hyperplasia, with ballooning degeneration of keratinocytes, spongiosis, and eosinophilic cytoplasmic inclusions. The dermis was expanded by proliferation of atypical mesenchymal cells with cytoplasmic inclusions. Additional findings included pulmonary adenomatous hyperplasia with cytoplasmic inclusions, renal tubular epithelial hyperplasia with cytoplasmic inclusions, atypical mesenchymal proliferation in the liver, and atypical mesenchymal proliferation with cytoplasmic inclusions in the seminal vesicles. Ultrastructurally, poxviral particles were observed in skin scrapings and sections of cutaneous and pulmonary nodules. Polymerase chain reaction targeting the highly conserved *Leporipoxvirus* DNA polymerase gene was positive using DNA extracted from the cutaneous lesions of all 3 squirrels. Nucleotide sequence of the 390 base PCR amplicons was closely related to that of other members of the genus *Leporipoxvirus*. To the authors' knowledge, this is the first report of cutaneous and systemic poxviral disease in American red squirrels with molecular characterization of the squirrel fibroma virus.

Key words: Gray squirrels; *Leporipoxvirus*; poxvirus; red squirrels; *Sciurus carolinensis*; squirrel fibromatosis; *Tamiasciurus hudsonicus*.

Squirrel fibromatosis (SF) is caused by a poxvirus closely related to the rabbit fibroma virus of the genus *Leporipoxvirus* in the subfamily *Chordopoxvirinae*.[6] This disease has been reported in eastern gray squirrels (*Sciurus carolinensis*),[9,16] a western gray squirrel,[17] and, presumptively, in a fox squirrel (*S. niger*).[5] In addition to the more common cutaneous form of the disease, a systemic form presenting as nodules in multiple viscera has also been reported.[10,23] Squirrel fibromatosis is characterized grossly by single or multiple, firm, alopecic dermal nodules and microscopically by typical poxviral lesions of epidermal hyperplasia, ballooning degeneration, and intracytoplasmic inclusion bodies. The most characteristic feature of SF is the proliferation of atypical fibroblasts in the superficial dermis. Infection is thought to be transmitted by biting arthropods, including mosquitoes and the squirrel flea *Orchopeus howardi*.[8] Squirrel fibromatosis has been reported from many states in the USA, most commonly along the eastern coast and infrequently in southeastern USA and in Ontario, Canada.[18] Squirrel fibromatosis generally occurs as a sporadic disease; epizootics are rare. An epizootic of SF in *S. carolinensis* was reported from Florida in 2002.[24] Like rabbit fibromatosis, SF usually regresses spontaneously. In a few fatal cases, death has been attributed to concomitant immunosuppression or disseminated poxviral disease.[18]

Squirrel fibroma virus (SFV) is classified as a member of the genus *Leporipoxvirus*, based on the similarity of the gross and histologic lesions in squirrels to those produced by rabbit fibroma virus in rabbits, immunological cross-reaction between squirrel fibroma virus and rabbit fibroma virus, and ultrastructure of the virus.[4] Although the complete genomic sequences of a number of poxviruses, including rabbit fibroma virus and rabbit myxoma virus, of the genus *Leporipoxvirus* are now available, there is no published report regarding genetic information of SFV.

In contrast to *S. carolinensis*, American red squirrels (*Tamiascuirus hudsonicus*) are small tree-dwelling squirrels distributed in the Appalachian mountain ranges of the northeastern USA and parts of Canada.[15] Once hunted for their fur, these squirrels are distinct from gray squirrels and European red squirrels (*Sciurus vulgaris*). Although a novel and fatal poxviral disease poses extreme threat to European red squirrels,[22,25,26] poxviral disease has not been previously reported in American red squirrels. In this communication, we report SF and systemic poxviral disease in 2 American red squirrels and 1 gray squirrel. We also describe the application of molecular biological techniques in the diagnosis of SF and provide insight into the molecular phylogeny of 3 SFV isolates.

From September 2005 through October 2006, 2 juvenile male red squirrels (squirrel Nos. 1 and 2) and 1 juvenile male black (gray) squirrel (*S. carolinensis*) were submitted for necropsy to Purdue University Animal Disease Diagnostic Laboratory. The squirrels

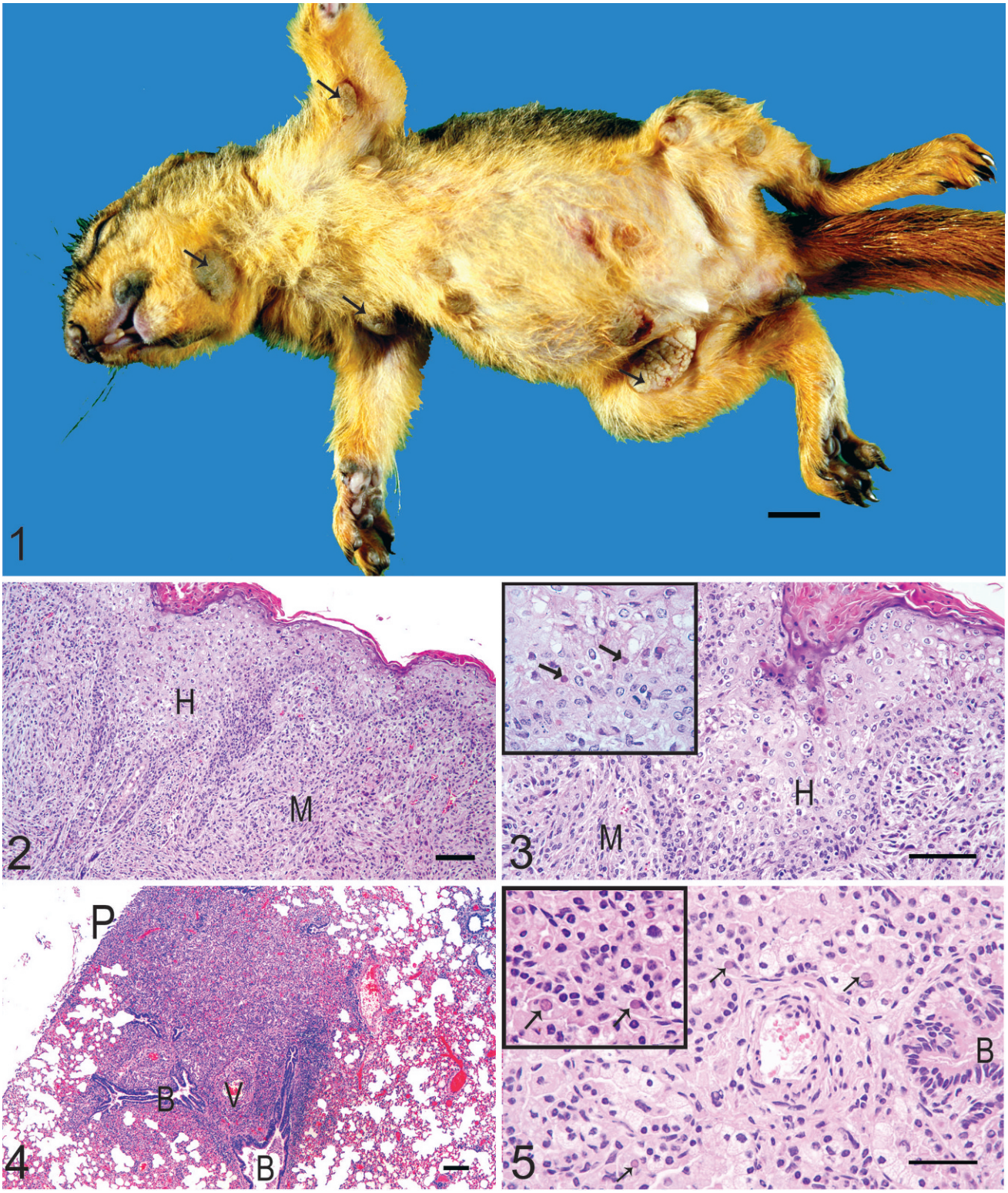

Fig. 1. Squirrel No. 1. Firm, crusted, alopecic dermal nodules (arrows) involving multiple areas of the body. Bar = 1 cm.

Fig. 2. Skin; squirrel No. 1. Dermal nodule with epidermal hyperplasia (H), ballooning degeneration, and marked atypical mesenchymal proliferation (M) in the dermis. HE. Bar = 100 μm.

Fig. 3. Skin; squirrel No. 1. Higher magnification of Figure 2 showing ballooning degeneration in epidermal keratinocytes, epidermal hyperplasia (H), and dermal mesenchymal proliferation (M). HE. Bar = 100 μm. *Inset*:

were reportedly found moribund or dead near urban areas in Elkhart County of northern Indiana. According to the submitter (a wildlife rehabilitator), increased numbers of dead or debilitated squirrels were reported near urban areas.

All 3 squirrels were in fair to good postmortem condition. Squirrel No. 1 weighed 120 g, squirrel No. 2 weighed 300 g, and squirrel No. 3 weighed 160 g. In squirrel No. 1, multiple firm, raised, dry to moist, crusted, 0.5–2.5-cm diameter nodules were in the skin around the eyes, ears, lips, trunk, feet, and genitalia (Fig. 1). A few nodules had hemorrhagic and ulcerated margins. The liver had multiple pale tan to white, 1–4-mm diameter, raised nodules; the lungs had disseminated pale gray to white nodules, up to 3 mm in diameter. Both kidneys in squirrel No. 1 were diffusely pale tan to off-white.

In squirrel No. 2, multiple 0.3–2.5-cm diameter, firm, raised, pale pink to white alopecic nodules were around the nose, lips, ears, and eyes, as well as on the head, neck, shoulders, trunk and feet. A 0.3-cm diameter pale tan to gray, firm plaque with pink-red margins was on the dorsal surface of the left lung. Multiple 0.1–0.15-cm diameter foci of similar color and consistency were in the right lung.

In squirrel No. 3, a 1.5-cm diameter alopecic dermal nodule with crusted, gray brown center, was in the right flank. Multiple 0.2–1.0-cm diameter, raised, firm nodules were on the right pinna, right shoulder, right foot, and left jaw. The left humerus had a complete transverse fracture; multiple fractures were in the left parietal and frontal bones. These fractures were attributed to trauma of unknown origin.

Multiple tissues including cutaneous nodules were fixed in 10% buffered formalin and processed routinely for histopathology. Five-micron-thick paraffin sections were stained by HE and examined microscopically. Histologically, dermal nodules in all 3 squirrels were characterized by marked parakeratotic hyperkeratosis and epidermal hyperplasia (acanthosis) with ballooning degeneration and single oval to round, 10–20-μm eosinophilic cytoplasmic inclusions in keratinocytes of the stratum spinosum (Fig. 2). All dermal nodules had proliferation of atypical fibroblast-like mesenchymal cells, many of which contained cytoplasmic poxviral inclusions (Fig. 3). The atypical mesenchymal cells/plump fibroblasts were arranged in bundles; mitotic activity was low (0–1 mitotic figure per high power [400×] field). In a few nodules, few neutrophils, lymphocytes, and macrophages infiltrated the collagenous connective tissue stroma. Ulcerated or crusted nodules contained multiple intracorneal pustules and colonies of bacterial cocci. In squirrel Nos. 1 and 2, the pulmonary nodules were composed of adenomatous proliferation of alveolar epithelial cells, which frequently contained eosinophilic cytoplasmic inclusion bodies (Figs. 4, 5). Additional histologic findings in squirrel No. 1 included atypical mesenchymal proliferation of loose connective tissue in portal areas in the liver, multifocal interstitial fibrosis with tubular epithelial proliferation and cytoplasmic inclusions in the kidney, and multifocal atypical mesenchymal cell proliferation with poxviral inclusions in the seminal vesicle.

For ultrastructural study, 1 mm^3 fragments of fresh tissues (skin and lung) were fixed in 3% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated in ethanol, and embedded in Epon. Ultrathin sections (70–90 nm) were contrasted with lead citrate and uranyl acetate, and examined with a Philips 201 electron microscope. Ultrastructurally, virus particles with typical features of poxvirus were observed in the cytoplasm of the epidermal keratinocytes and dermal fibroblasts from cutaneous nodules (Fig. 6) and in the proliferative epithelial cells in the lung of squirrel No. 1. Viral particles measured 200 × 300 nm and contained an envelope and an electron dense, biconcave nucleocapsid core surrounded by lateral bodies.

For molecular diagnostics, lesional tissue was homogenized using tissue PowerGen homogenizer (Fisher Scientific Pittsburgh, PA) in phosphate buffered saline. From the homogenate, total deoxyribonucleic acid (DNA) was isolated using the Qiagen DNeasy tissue kit (Qiagen, Valencia, CA). Five hundred nanograms of DNA was used for polymerase chain reaction (PCR) amplification, using primers designed to target the DNA polymerase gene of the rabbit fibroma virus (genus *Leporipoxvirus*).[2] Although poxviral genera within both subfamilies of the family *Poxviridae* have extensive nucleotide sequence divergence among them, a few evolutionarily conserved genes have been used for phylogenetic comparison of various poxviruses.[12] Poxviral DNA polymerase is an essential gene for poxviral replication in host cell cytoplasm and is expected to be highly conserved among closely related poxviruses. Samples of DNA isolated from the skin of an unrelated red squirrel and from a pig with swinepox were used as negative controls. The PCR products were electrophoresed in 1.5% agarose gel and visualized under ultraviolet light. Specific 390-bp amplicons were excised from the agarose gel, purified using Geneclean III kit

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Higher magnification of epidermis with swollen keratinocytes, cytoplasmic vacuolation, and cytoplasmic poxviral inclusions (arrow).

Fig. 4. Lung; squirrel No. 1. Focal adenomatous nodule in the pulmonary parenchyma. Also note the bronchiole (B), blood vessel (V), and the pleura (P). HE. Bar = 100 μm.

Fig. 5. Lung; squirrel No. 1. Higher magnification of the pulmonary nodule in Figure 4 showing hypertrophic and hyperplastic alveolar epithelial cells with cytoplasmic inclusions (arrows). HE. Bar = 100 μm. *Inset*: Higher magnification showing cytoplasmic poxviral inclusion in an alveolar epithelial cell.

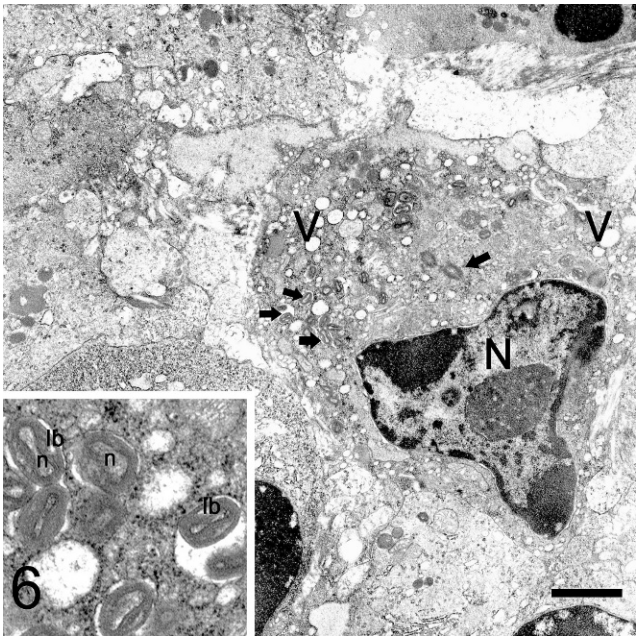

Fig. 6. Skin; squirrel No. 1. Transmission electron micrograph showing a keratinocyte with cytoplasmic vacuolation and numerous brick-shaped electron-dense poxviral particles (arrows) in the cytoplasm. N = nucleus, V = cytoplasmic vacuoles. Uranyl acetate and lead citrate. Bar = 400 nm. *Inset*: Cytoplasmic poxviral particles with central core containing the nucleocapsid (n) surrounded by lateral bodies (lb).

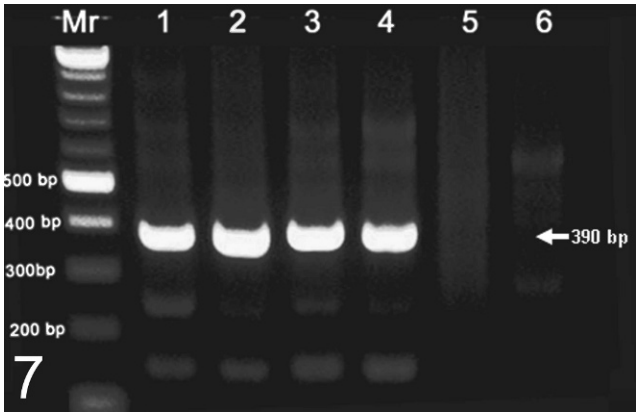

Fig. 7. Agarose gel electrophoresis of PCR products following amplification of DNA samples using primer pairs targeting the DNA-dependent DNA polymerase gene of *Leporipoxvirus*. Skin (lane 1) and lung (lane 2) from squirrel No. 1, skin from squirrel No. 2 (lane 3), skin from squirrel No. 3 (lane 4), normal squirrel skin (lane 5), and skin from a pig with swine pox (lane 6). Mr = DNA ladder. A band corresponding to specific 390 bp PCR amplicon is observed in squirrels with fibromatosis (lanes 1–4).

(MP Biomedical, Solon, OH), and sequenced at Purdue University Genomic Core Facility. The overlapping sequences generated by both primers (284 bp) were compared with related virus sequences for phylogenetic analysis.[21] *Leporipoxvirus*-specific 390-bp PCR amplicons corresponding to the DNA-dependent DNA polymerase gene were obtained from DNA samples from dermal nodules of all 3 squirrels and the lung of squirrel No. 1 (Fig. 7, lanes 1–4). DNA from the skin of an unaffected red squirrel and a pig with swinepox did not amplify the target sequence (lanes 5 and 6).

Lesion distribution in all 3 squirrels was typical of previously reported cases of SF.[18] Multiple nodules may be attributed to viremia or multiple exposures to the transmission vector. Viremia is also associated with the development of lesions in visceral tissues such as lungs. In the present case, systemic poxviral disease was suspected in squirrel Nos. 1 and 2, with more extensive involvement of lung in squirrel No. 1. Pulmonary lesions in squirrel Nos. 1 and 2 are reminiscent of the adenomatous lesions that develop in suckling squirrels following intratracheal inoculation of SFV.[11] Disseminated infection following natural infection with SFV has been previously described.[8,10,16] Juvenile squirrels are most susceptible to disseminated or fatal disease. Although not extensively investigated, immunological incompetence of juvenile squirrels has been proposed as an important reason for their susceptibility to disseminated disease or for developing multiple fibromas.[9,16] The pathogenesis of disseminated poxviral disease is not completely understood, but viremic spread is considered the most likely mechanism.[16] Squirrel fibroma virus tumorigenesis has not been studied extensively; however, fibromatosis appears to be species-specific phenomenon.[13] Following intradermal or intratracheal inoculation of squirrel fibroma virus in suckling squirrels and rabbits, characteristic dermal fibromas or pulmonary adenomatous lesions developed only in the former.[11]

The genome has been partially or completely sequenced for a number of poxviruses.[14] However, molecular genetic or genomic information on SFV has not been published. In the present study, we developed a PCR assay for specific detection of *Leporipoxvirus* DNA in tissue samples. The PCR amplicons generated by primers targeting the viral DNA polymerase gene were sequenced, and 284-bp nucleotide sequences thus generated were submitted to GenBank (accession nos. EU732592, EU732593, and EU729367) and phylogenetically compared with 9 different chordopoxviruses. Poxviruses selected for comparison included members of the genus *Leporipoxvirus* (rabbit fibroma virus: GenBank accession NC_001266, and rabbit myxoma virus: GenBank accession NC_001132), genus *Parapoxvirus* (orf virus: GenBank accession NC_005336), *Orthopoxvirus* (cowpox virus: GenBank accession NC_003663, vaccinia virus: GenBank accession NC_006998, and ectromelia virus: GenBank accession NC_004105), genus *Suipoxvirus* (GenBank accession NC_003389), and genus *Capripoxvirus* (sheep poxvirus: GenBank accession NC_004002, and lumpy skin disease virus: GenBank accession NC_003027). The sequence alignment was performed with the MegAlign application

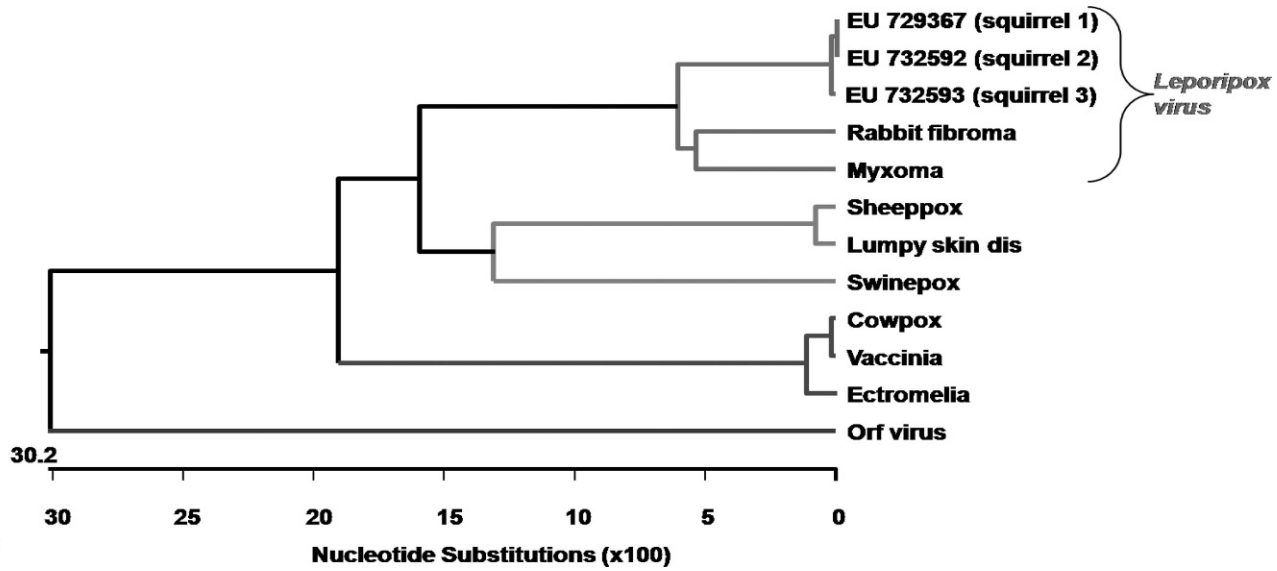

Fig. 8. Phylogenetic comparison of poxviruses. Phylogenetic tree based on 284 bp nucleotide sequence of DNA-dependent DNA polymerase gene of squirrel poxviruses from the present study (GenBank accession numbers EU 729367, 732592, and 732593) and homologous sequence from other poxviruses. Gene sequences for other poxviruses were retrieved from the GenBank. The tree is drawn by the neighbor-joining method.

of the Lasergene software (DNASTAR Inc., Madison, WI) using the clustalW method, and the phylogenetic tree was drawn by the neighbor-joining method[21] based on the partial (284 bp) nucleotide sequence of poxviral DNA polymerase gene of SFVs (present study) and other poxviruses (Fig. 8). All 3 SFVs clustered together and were most closely related to the rabbit fibroma virus and Shope fibroma virus of the genus *Leporipoxvirus*. Squirrel fibromaviruses from the present study showed 90.1% nucleotide identity with rabbit fibroma virus and rabbit myxoma virus based on the partial nucleotide sequence of the DNA polymerase gene.

Based on the characteristic gross and histologic lesions and demonstration of viral particles by electron microscopy, SF was diagnosed in 2 red squirrels and 1 gray squirrel. Systemic poxvirus disease was diagnosed in the 2 red squirrels (Nos. 1 and 2) based on involvement of multiple tissues, demonstration of poxvirus particles in cutaneous fibromas and the lung by electron microscopy, and detection of poxviral DNA by PCR targeting the leporipoxvirus DNA polymerase gene. To our knowledge, this is the first study demonstrating molecular relatedness of SFVs to rabbit fibroma virus and rabbit myxoma virus.

Although the gross skin lesions of SF are typically diagnostic, other poxviral diseases should be considered for extensive cutaneous lesions observed in squirrels. Another deadly poxviral disease of squirrels is caused by a novel poxvirus that was previously thought to be a parapoxvirus.[22] However, recent molecular characterization of the poxvirus has revealed that it is not a *Parapoxvirus*; it has been proposed as a distinct genus under the family *Chordopoxvirinae*.[16] This fatal disease of European red squirrels (*S. vulgaris*) is widespread in many parts of Europe[20,26]; it has become a major threat to the survival of *S. vulgaris* and has stimulated extensive research efforts in Britain. Although gray squirrels in affected areas seroconvert, clinical disease in *S. carolinensis* has been reported only once.[3] According to 1 serologic survey, more than 60% of *S. carolinensis* in the UK had antibodies against this novel poxvirus in comparison with only 2.9% of *S. vulgaris*.[19] Although this disease has never been reported in the USA, serum samples collected recently from 7 gray squirrels in Dane County, Wisconsin, tested positive for antibodies against this novel poxvirus, suggesting the possibility of its presence.[14] Cutaneous fibromatosis and systemic poxviral disease in squirrels in the present study are distinct from the deadly poxviral disease of European red squirrels. In SF, single or multiple cutaneous or visceral nodules (fibromas) generally develop. On the other hand, the novel squirrel poxviral disease of the UK is characterized by the formation of severe cutaneous ulceration with hemorrhagic scabs around the eyes, nose, and mouth.[16,18] Another poxviral disease of squirrels is the zoonotic disease monkeypox.[7,13,18] Natural and experimental disease caused by monkeypox virus leading to extensive cutaneous lesions has been described in African squirrels, including *Heliosciurus rufobrachium*, *H. gambianus*, *Funisciurus anerythrus*, and *F. lemniscatus*.[7,13,18] High seroprevalence of monkeypox virus antibodies was reported in *F. anerythrus*. Monkeypox virus infection, however, has not been reported in squirrels in North America. Lesions of poxviral diseases often overlap, necessitating the use of molecular diagnostic for definitive diagnosis. Definitive diagnosis of a poxviral disease in squirrels will be facilitated by the availability of the *Leporipoxvirus*-specific PCR assay described in this report.

An interesting epidemiological feature of the cases presented here is the prevalence of SF in this area over a 3-year period. Squirrels in this area have experienced

increased morbidity; we recently reported acute disseminated toxoplasmosis in juvenile red squirrels in this area.[1] The epidemiologic features and short- or long-term implications of SF on squirrels in this area are not known, but the continued prevalence of the disease warrants further investigation. A variety of causal possibilities may be considered as suggested by Terrell et al. who reported the epizootic of SF in Florida.[24] These include increased vector prevalence and introduction of a novel strain of SFV. We did not find differences in the partial nucleotide sequence of the DNA polymerase gene of SFVs in the present study, but genetic characterization of additional new or archived SF tissue material may be helpful in delineating genetic differences in various SFV isolates.

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