

2009

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Brown, Charles R.; Padhi, Abinash; Moore, Amy T.; Brown, Mary Bomberger; Foster, Jerome E.; Pfeffer, Martin; O'Brien, Valerie A.; and Komar, Nicholas, "Ecological divergence of two sympatric lineages of Buggy Creek virus, an arbovirus associated with birds" (2009). *Papers in Natural Resources*. 474.

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Ecological divergence of two sympatric lineages of Buggy Creek virus, an arbovirus associated with birds

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Abstract. Most arthropod-borne viruses (arboviruses) show distinct serological subtypes or evolutionary lineages, with the evolution of different strains often assumed to reflect differences in ecological selection pressures. Buggy Creek virus (BCRV) is an unusual RNA virus (Togaviridae, *Alphavirus*) that is associated primarily with a cimid swallow bug (*Oeciacus vicarius*) as its vector and the Cliff Swallow (*Petrochelidon pyrrhonota*) and the introduced House Sparrow (*Passer domesticus*) as its amplifying hosts. There are two sympatric lineages of BCRV (lineages A and B) that differ from each other by >6% at the nucleotide level. Analysis of 385 BCRV isolates all collected from bug vectors at a study site in southwestern Nebraska, USA, showed that the lineages differed in their peak times of seasonal occurrence within a summer. Lineage A was more likely to be found at recently established colonies, at those in culverts (rather than on highway bridges), and at those with invasive House Sparrows, and in bugs on the outsides of nests. Genetic diversity of lineage A increased with bird colony size and at sites with House Sparrows, while that of lineage B decreased with colony size and was unaffected by House Sparrows. Lineage A was more cytopathic on mammalian cells than was lineage B. These two lineages have apparently diverged in their transmission dynamics, with lineage A possibly more dependent on birds and lineage B perhaps more a bug virus. The long-standing association between Cliff Swallows and BCRV may have selected for immunological resistance to the virus by swallows and thus promoted the evolution of the more bug-adapted lineage B. In contrast, the recent arrival of the introduced House Sparrow and its high competence as a BCRV amplifying host may be favoring the more bird-dependent lineage A.

Key words: *alphavirus; Cliff Swallow; disease emergence; host–pathogen dynamics; House Sparrow; infectious disease; Oeciacus vicarius; Passer domesticus; Petrochelidon pyrrhonota; vector biology; virus ecology; virus evolution.*

INTRODUCTION

The rapid evolution of RNA viruses presents many opportunities for virus adaptation to new hosts, ecological environments, and transmission strategies.

Manuscript received 17 September 2008; revised 27 January 2009; accepted 10 February 2009. Corresponding Editor: K. Wilson.

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Because they lack proofreading capability in their polymerases during replication, these viruses have the highest mutation rates known (Scott et al. 1994, Moya et al. 2004) and consequently have increased potential for evolutionary change in response to host defenses. Although factors such as large virus population sizes and the necessity for some viruses to replicate in both an invertebrate vector and a vertebrate host may also constrain virus evolution (Weaver et al. 1992, Weaver 2006), nevertheless RNA viruses exhibit great genetic variability within and between populations. This variability is reflected in many arthropod-borne viruses (arboviruses) showing distinct serological subtypes or evolutionary lineages that correlate with extent of virulence, host and vector associations, or geography (Ewald 1994, Holmes and Burch 2000, Weaver and Barrett 2004).

Despite considerable work in describing and characterizing virus subtypes and lineages both serologically and phylogenetically, little is understood about the

ecological factors that promote arbovirus diversification. The conventional wisdom for viruses in general is that allopatric lineages reflect chance dispersal across geographic or climatic barriers followed by genetic drift and/or gradual evolutionary change through random mutations (e.g., Lindsay et al. 1993, Scott et al. 1994, Bourhy et al. 1999, Nadin-Davis et al. 1999, Zhang et al. 2005), or that different virus lineages reflect adaptation to different amplifying hosts that vary in their immunological competence and/or relative mobility and thus their propensity to mix virus strains (e.g., birds vs. small mammals; Weaver et al. 1992, 1994, 1997, Poidinger et al. 1997, Brault et al. 1999, Bowen et al. 2000, Gould et al. 2001, Weaver and Barrett 2004, Anishchenko et al. 2006, Weaver 2006). Other work has suggested that different virus strains may be specific to certain mosquito vectors (e.g., Powers et al. 2000; see Schuffenecker et al. 2006), presumably coevolving with their respective species to maximize transmission under certain ecological conditions. Yet, most of what we understand about the evolution of arbovirus lineages is based on assumptions about virus ecology, and studies that systematically investigate the extent of ecological differences between sympatrically occurring arbovirus lineages or subtypes in the field are lacking. By documenting such differences, we can better understand selective environmental pressures that may lead to arbovirus evolution and potentially the emergence of new pathogens (Scott et al. 1994, Holmes and Burch 2000).

In this study, we explore how two sympatric arbovirus lineages may be diverging ecologically. We examine specifically whether the different lineages are likely to be associated with different vertebrate hosts, whether they potentially differ in virulence and peak time of seasonal occurrence, whether they respond differently to the ecology of their hosts, whether the behavior of their insect vectors potentially varies, and whether genetic diversity of these lineages varies with ecological conditions. If such differences occur, we may be seeing virus adaptation and the emergence of new virus strains.

Buggy Creek virus (BCRV; *Togaviridae*, *Alphavirus*) is a single-strand, positive-sense RNA virus antigenically and phylogenetically placed within the western equine encephalomyelitis virus (WEEV) complex (Calisher et al. 1980, Hopla et al. 1993, Powers et al. 2001, Pfeffer et al. 2006). BCRV is unusual among alphaviruses because it is vectored primarily by an ectoparasitic swallow bug (Hemiptera: Cimicidae: *Oeciacus vicarius*), rather than by mosquitoes. Swallow bugs are closely associated with the colonially nesting Cliff Swallow (*Petrochelidon pyrrhonota*) and the introduced House Sparrow (*Passer domesticus*) that occupies nests in Cliff Swallow colonies. The bugs live primarily in the swallow nests throughout the year, and consequently they can be predictably located and sampled for virus at any time, even when birds are not present (Brown et al. 2001, 2008, 2009a, Strickler 2006, Moore et al. 2007). The bugs take blood meals from both Cliff Swallows and House Sparrows,

and in this way potentially transmit the virus to vertebrate amplifying hosts.

In this study, we used sequence data from this virus's E2 glycoprotein-coding region (Pfeffer et al. 2006, Brown et al. 2008) to designate the lineage for 385 BCRV isolates collected from bug vectors in 1998–2006 at a study area in southwestern Nebraska, USA where Cliff Swallows and swallow bugs have been studied in the field for over 25 years (e.g., Brown and Brown 1996). The E2 gene in alphaviruses codes for a glycoprotein that is responsible for cell receptor binding (Navaratnarajah and Kuhn 2007) and is the region of the genome most sensitive to selection brought about by the immune systems of different hosts (Strauss and Strauss 1994, Powers et al. 2001, Pfeffer et al. 2006). The E2 region also determines infection of invertebrate vectors (Brault et al. 2002). If there are functional differences among virus isolates that reflect varying levels of adaptation to cell receptors of different hosts or vectors, these differences may be reflected in the E2 gene.

METHODS

Study organisms

Buggy Creek virus was first isolated in 1980 from swallow bugs collected at a Cliff Swallow colony along Buggy Creek in Grady County, west central Oklahoma, USA (Hopla et al. 1993). BCRV and another alphavirus, Fort Morgan virus (FMV), which is also associated with Cliff Swallows and swallow bugs (Hayes et al. 1977, Calisher et al. 1980, Scott et al. 1984), are strains of the same virus (Pfeffer et al. 2006, Padhi et al. 2008). The two lineages of Buggy Creek virus (lineages A and B) differ from each other by >6% at the nucleotide level (Pfeffer et al. 2006).

Cliff Swallows are highly colonial passerines that breed throughout most of western North America (Brown and Brown 1995). They build gourd-shaped mud nests and attach them to the vertical faces of cliff walls, rock outcrops, or artificial sites such as the eaves of buildings, bridges, and highway culverts. Cliff Swallows are migratory, wintering in southern South America, and have a relatively short breeding season in North America, from late April to late July. Most birds raise only one brood.

House Sparrows were introduced into North America from Europe in the 1800s and are found in all parts of the United States (Lowther and Cink 1992). Sparrows usurp Cliff Swallow nests and will occupy them until the nests fall from the substrate. Numbers of sparrows vary among colony sites, with some colonies having none and others having only sparrows. House Sparrows are nonmigratory and resident around the swallow colonies throughout the year. Many individuals raise up to three broods per summer at our study site.

The hematophagous swallow bug is an ectoparasite primarily of Cliff Swallows. Swallow bugs are nest-based parasites (in the bedbug family) that overwinter in the birds' nests or in the cracks and crevices of the

nesting substrate near the nests. Infestations can reach 2600 bugs per nest, and the bugs affect many aspects of Cliff Swallow life history (Brown and Brown 1986, 1992, 1996, Chapman and George 1991, Loye and Carroll 1991). Swallow bugs begin to reproduce as soon as they take a blood meal in the spring, usually at about the time the Cliff Swallows first arrive at a site. Eggs are laid in several clutches that hatch over variable lengths of time, ranging from 3–5 days (Loye 1985) to 12–20 days (Myers 1928). Nymphs undergo five molts before becoming adults, requiring a blood meal for each molt, and are mature about 10 weeks after hatching (Loye 1985). Cliff Swallows do not use all of the colony sites in a given year (Brown and Brown 1996), and some bugs can survive at a site for up to three years in the absence of any bird hosts (Smith and Eads 1978, Rannala 1995), although there is substantial mortality after the first year of host absence (C. Brown et al., *unpublished data*). The bugs readily parasitize House Sparrows nesting in Cliff Swallow colonies, but they seem to prefer swallows as hosts (V. O'Brien and C. Brown, *unpublished data*). Swallow bugs disperse between nests within a colony by crawling on the substrate and between colony sites by clinging to the feet and legs of Cliff Swallows that move from one site to another (Brown and Brown 2004a).

Relatively little work has been done on swallow bugs as vectors for BCRV. Early studies on the FMV strain of BCRV showed that up to 80% of bugs became infected after feeding on virus-infected House Sparrows in the laboratory and that the transmission rate of the virus from bugs to uninfected birds was 29% (Rush et al. 1980). Swallow bugs that fed on birds that had been infected by other bugs in turn became infected, demonstrating a complete transmission cycle (Rush et al. 1980). BCRV has been isolated from the bugs' salivary glands (Rush et al. 1981); no other tissues have been tested for virus. Bugs maintained virus for up to 311 days after being infected and were capable of infecting new hosts at that time (Rush et al. 1980).

Study site

Our study site is centered at the Cedar Point Biological Station (41°13' N, 101°39' W) near Ogallala, in Keith County, along the North and South Platte Rivers, southwestern Nebraska, USA, and also includes portions of Lincoln, Deuel, Garden, and Morrill counties. Cliff Swallows have been studied there since 1982. Approximately 170 Cliff Swallow colony sites are in the 200 × 60 km study area; about a third of these are not occupied by swallows in a given year. In our study area, Cliff Swallow colony size ranges from two to 6000 nests, with some birds nesting solitarily. Over a 25-year period, colony size ($n = 1812$) was 393 ± 15 (mean \pm SE) nests. House Sparrow colony size has been less well monitored but typically varies between one and 20 nests in our study area. Each colony site tends to be separated from the next nearest by 1–10 km but in a few cases by ≥ 20 km. The study area is described in detail by Brown

and Brown (1996). Cliff Swallow colonies in this study were situated on either large highway bridges that spanned the North or South Platte Rivers or in box-shaped culverts underneath roads or railroads. Relative to bridges, culverts were much lower in height, typically more humid, darker, and varied less in temperature during all seasons.

Field collections of bugs

Collections of swallow bugs for virus isolation in the study area were made each summer (from May to August) between 1998 and 2006. At sites with active Cliff Swallow nests, swallow bugs were taken from the outsides of the nests by brushing bugs off nests into a wide-mouthed collecting jar. At inactive colony sites, we removed swallow nests to expose bugs on the substrate behind the nests and harvested additional bugs by sorting through the mud nest fragments. Lineage distributions were unaffected by whether bugs were brushed off nests at active sites or harvested from collected nests; for example, in years when both active and inactive colony sites were sampled, 38.5% of isolates at active sites were lineage A and 45.4% at inactive sites, a nonsignificant difference ($\chi^2_1 = 0.58$, $P = 0.44$). Bugs were sorted into pools of 100 individuals while alive and frozen immediately at -70°C . We sampled bugs from throughout a colony site. For each collection, we recorded date, where on the nest the bugs were collected, and nest status (whether Cliff Swallows were present). For each colony site, we recorded colony size (number of active Cliff Swallow nests) and whether House Sparrows were present anywhere in the colony. Colony-site age was scored as old (>25 years) if the site was present and was being, or had been, used by Cliff Swallows when our study started in 1982. Sites were scored as young (0–11 years old) if the colony site (bridge or culvert) was built and birds started using it for the first time between 1995 and 2006. There were no colony sites between 11 and 25 years old. We did not separate adult and instar bugs in our analyses, as earlier work showed no effect of bug age on BCRV prevalence in these insects (Brown et al. 2001). Additional details on field sampling and collecting are given in Moore et al. (2007).

For analyses that examined lineage distributions per colony site, we used data (when available) for 20 colony sites where we had greater than five virus isolates identified to lineage. At other sites, we had five or fewer isolates, principally because virus infection of bugs varies among sites, and some colonies have low infection rates (e.g., Brown et al. 2001, 2007). For analyses that examined lineage distributions using binary independent variables (e.g., culvert or bridge, sparrows present or not), we used isolates from all sites including colonies with five or fewer isolates.

Estimating bird movement

To examine whether the distributions of the lineages at colony sites potentially varied with the extent of Cliff

Swallow movement, we estimated the overall likelihood of a swallow moving into a site from all other colony sites in the study area (Brown et al. 2007). Using extensive mark–recapture data available for Cliff Swallows in the study area (e.g., Brown and Brown 2004b), we used multi-state statistical analyses (Lebreton and Pradel 2002) to estimate the probability that an individual immigrated (at least transiently) into a colony from another colony in the study area per any 2-day interval during the summer nesting season. We designated each capture as either present at the focal colony (state 1) or present at any other colony in the study area (all combined into state 2), and estimated movement between the focal colony and all other colonies in each year. For the analyses here, the yearly movement probabilities were averaged to get an overall estimate of movement into a given colony in the years it was active. Movement was measured by the parameter, ψ (\pm SE), which specifically describes the probability of an individual making the given transition between the specified colonies. Movement in these analyses reflects both the daily travels of transient, nonbreeding birds between colonies (perhaps while they are assessing where to nest) and the potential dispersal of breeding individuals elsewhere following a successful or unsuccessful nesting attempt. Program MARK (White and Burnham 1999) was used to generate maximum-likelihood estimates of movement probabilities. Further details on the movement analyses, including descriptions of the models used and model-fitting results, are given in Brown et al. (2007).

Virus isolation and determining lineages

We determined lineages for 385 BCRV isolates (all from bugs). Virus was isolated from bug pools following the methods given in Brown et al. (2008). RNA from each isolate was used in an alphavirus reverse-transcription polymerase chain reaction (RT-PCR) to amplify the entire 1269 base pairs of the E2 gene (see Brown et al. 2008). Sequences were aligned against the corresponding region in a 1981 BCRV reference sequence (strain 81V1822, GenBank no. AF339474) and fragments combined for a given isolate using SeqMan 6.1 (Lasergene, DNASTar, Madison, Wisconsin, USA) to obtain a contiguous nucleotide sequence for each sample. Sequences generated from this study are deposited in GenBank (accession numbers EU483667–EU484043). The lineage of each isolate was determined from maximum likelihood and Bayesian inference phylogenies constructed using PAUP* 4.0b10 (Swofford 2002) and MrBayes Version 3.1 (Huelsenbeck and Ronquist 2001). The number of unique sequences of a lineage was determined using Collapse version 1.2 (Posada 2004). Arlequin version 3.1 (Schneider et al. 2000) was used to estimate the haplotype diversities of each lineage at a site. Each unique sequence that differed from all others by at least one nucleotide change was designated as a haplotype. Further details on methods of sequencing

and lineage determination are given in Brown et al. (2008) and Padhi et al. (2008).

Each BCRV isolate is actually a sample of multiple and potentially variable virus particles within the host (Domingo 1998, Pfeffer et al. 2006); in our study, an isolate from a given sample likely represents the dominant genotype present. In three cases where an isolate had evidence of a sequence polymorphism, indicating the presence of multiple viral genotypes, it was excluded from analysis.

Determining cytopathicity and viral RNA concentration

Bug homogenates that were initially positive from RT-PCR screening were subjected to plaque assay on Vero cells. We added 100 μ L of the supernatant from the swallow bug homogenate in duplicate to a monolayer of Vero cells in a six-well cell-culture plate, incubated it for 1 h at 37.8°C in 5% CO₂, and then overlaid each monolayer with 3 mL 0.5% agarose in M-199 medium supplemented with 350 mg/L sodium bicarbonate, 29.2 mg/L L-glutamine, and antibiotics and returned the plate to the incubator. A second overlay containing 0.004% neutral red dye was added after 2 days' incubation for plaque visualization. Plaques were scored daily for 5 days. Isolates that formed plaques on Vero cells were considered to contain cytopathic virus. For samples that showed no plaques on Vero cells, we re-extracted RNA from the remaining swallow bug homogenate and performed another RT-PCR assay. Those not forming plaques but that were positive in the second RT-PCR were considered to represent non-cytopathic virus (Moore et al. 2007).

To determine the relative viral RNA concentration of each isolate, we designed a one-step multiplex real-time RT-PCR assay (Gentle et al. 2001, Pfaffl 2001, Marino et al. 2003). Quantitative RT-PCR requires that the data be normalized to account for the variability involved in the reverse transcription of RNA and thus to ensure assay reproducibility (Bustin and Nolan 2004, Huggett et al. 2005). We used an externally added control RNA for this purpose (Gilsbach et al. 2006), as the pools of bugs had no inherent internal control suitable for quantitative RT-PCR. Samples were prepared for real-time RT-PCR by mixing 3 μ L of each isolate with an equal volume of 20 ng/ μ L total human RNA (diluted from a 50 ng/ μ L stock of TaqMan Control Total RNA, which was stored frozen in aliquots; Applied Biosystems, Foster City, California, USA). One-step quantitative RT-PCR was then performed using a QuantiTect Probe RT-PCR kit (Qiagen, Valencia, California, USA). The manufacturer's recommended protocol for custom assays was used except that a concentration of 0.2 μ mol/L was used for each of the four sequence-specific primers in the multiplex reaction. For each reaction, 2.5 μ L of the combined RNA sample was used as template in 25- μ L reactions and cycled in a Cepheid SmartCycler (Cepheid, Sunnyvale, California, USA). LUX Primers 418RU (5'-CGTGC AATGGTG-

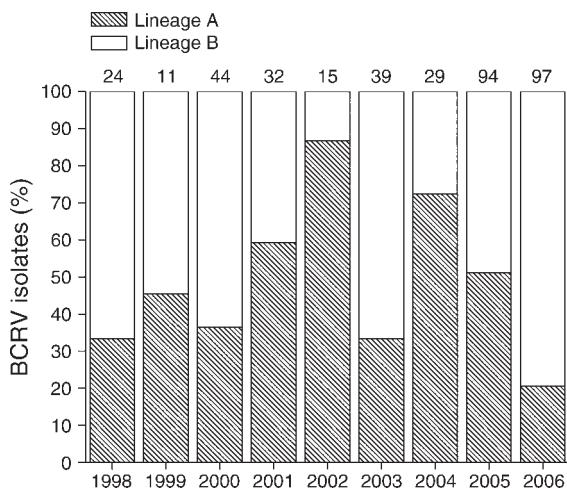


FIG. 1. Percentage of lineage A isolates (hatched bars) and lineage B isolates (open bars) among the total Buggy Creek virus (BCRV) isolates from bugs each year in the study area in southwestern Nebraska, USA. Sample sizes (total number of isolates sequenced) per year are given above the bars. Percentages of the two lineages varied significantly among years ($\chi^2_8 = 51.1$, $P < 0.00001$).

GAAATTGATA-3') and the FAM-labeled 406FL (5'-CAACAAGGTGCAGCGTAAGTTTG[FAM]TG-3') were derived from BCRV E2 nucleotide positions 418–439 and 387–406, respectively. JOE-labeled Certified LUX Primers that target the human beta-2-microglobulin (B2M) gene were obtained from Invitrogen (Carlsbad, California, USA). Melting curve analysis was performed on each sample at the end of cycling to verify the purity of amplicons.

To correct for variations in the efficiency of each RT reaction, the C_t value (cycle number at which the fluorescence exceeded a predefined threshold) obtained for the JOE-labeled B2M amplicon was subtracted from the C_t value obtained for the FAM-labeled BCRV amplicon for each reaction, thus giving a normalized C_t (ΔC_t) for each BCRV isolate measured (Gilsbach et al. 2006). As the ΔC_t value increased for a sample, the RNA concentration of that sample relative to others decreased.

RESULTS

Temporal differences

The percentage of lineage A among the Buggy Creek virus (BCRV) isolates each summer (Fig. 1) varied from 20.6% (in 2006) to 86.7% (in 2002). The relative proportions of the lineages differed significantly between years (Fig. 1), but there was no discernable trend for an increase or decrease over the years of the study. Combined over all summers ($n = 385$ isolates), lineage A represented 42.3% of isolates and lineage B, 57.7%; this ratio differed significantly from 50:50 (binomial test, $P = 0.001$).

When collection dates within a summer season were combined into two-week intervals across all years, beginning on 5 May and extending until 2 August, the percentages of lineages A and B in the intervals were significantly different (Fig. 2). Lineage A peaked about two weeks earlier than lineage B. Over 50% of all lineage B isolates were collected between 19 June and 3 July, whereas lineage A showed less temporal clustering within a summer (Fig. 2).

Colony site differences

In general, both lineages co-circulated at the same colony sites; of the 20 sites with more than five isolates identified to lineage from 1998 to 2006, we detected both lineages at 15 sites (75.0%). However, the proportion of the two lineages varied widely among sites, with lineage A ranging from 2.5% to 96.9% of the total isolates at a site. Colony-site age affected the likelihood of finding lineage A vs. lineage B at a site. Isolates from relatively young (recently colonized) sites ($n = 119$ isolates) were predominantly lineage A (84.9%; binomial test, $P < 0.0001$), whereas isolates from older, established colony sites that had been used by swallows for many years ($n = 183$ isolates) were mostly lineage B (90.2%; binomial test, $P < 0.0001$). The lineage distributions differed significantly between young and old colony sites ($\chi^2_1 = 170.0$, $P < 0.00001$). Substrate type also was associated with the likelihood of detecting lineage A vs. lineage B at a site. More of the isolates from culverts ($n = 213$ isolates) were of lineage A (61.5%) than of lineage B (38.5%; binomial test, $P = 0.0003$), whereas those from bridges ($n = 143$ isolates) were predominantly lineage B (90.2%; binomial test, $P < 0.0001$); the lineage distributions differed significantly between the two kinds of colony substrates ($\chi^2_1 = 94.8$, $P < 0.00001$).

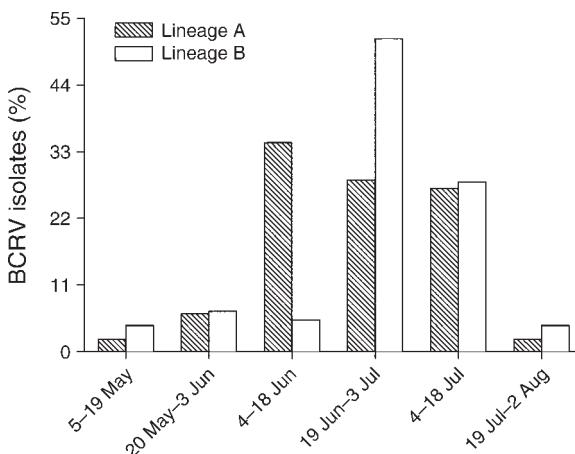


FIG. 2. Percentage of total isolates from bugs of BCRV lineage A (hatched bars, $n = 145$ isolates) and percentage of total isolates of lineage B (open bars, $n = 211$ isolates), collected during 15-day intervals across the summer for all years combined. The lineages differed significantly in their distributions across date intervals ($\chi^2_5 = 56.6$, $P < 0.0001$).

TABLE 1. Analysis of covariance (ANCOVA) to detect effects and interactions of variables potentially affecting the percentage of lineage A among Buggy Creek virus isolates at Cliff Swallow colony sites where more than five isolates were identified to lineage.

Variable	$F_{1,17}$	P
Colony age (young or old)†	0.35	0.57
Colony size‡	0.16	0.70
Substrate (bridge or culvert)	0.02	0.89
Sparrows (present or absent)§	8.28	0.016
Colony size × substrate	0.37	0.56
Colony size × colony age	0.55	0.48
Colony size × sparrows	9.16	0.013

† Young colonies were those 0–11 years in age; old colonies were those >25 years in age. There were no colony sites that were between 11 and 25 years old. Colonies were located in southwestern Nebraska, USA.

‡ Mean number of active Cliff Swallow nests at a site in years when it was used by swallows.

§ House Sparrows.

The proportions of lineages A and B were significantly associated with the presence of House Sparrows at Cliff Swallow colony sites. At colony sites with sparrows, 66.2% of isolates ($n = 133$) were of lineage A, compared to only 25.6% of the isolates at colony sites without sparrows ($n = 219$ isolates). The distribution of the lineages differed significantly between sites with and without House Sparrows ($\chi^2_1 = 56.4$, $P < 0.0001$).

Because of potential covariation between colony age, substrate type, and presence or absence of sparrows, we assessed the independent effect of these variables (plus colony size) on the proportion of lineage A at 18 colony sites (those with more than five isolates identified to lineage; Table 1). For these sites, the presence or absence of sparrows was the only significant predictor of the percentage of virus lineage A (Table 1). There was also a significant interaction between mean Cliff Swallow colony size in years that swallows used a site and presence of sparrows (Table 1). This interaction was brought about by a significant decrease in the proportion of lineage A at larger swallow colonies when sparrows were present but no significant relationship with colony size in their absence (Fig. 3).

Effect of bird movement

For 12 colony sites where we had estimates of the probability of a Cliff Swallow moving into the site per two-day interval during the summer, the proportion of lineage A among the isolates found at the site increased significantly with bird movement probability (Fig. 4). Lineage B was more likely to be found at sites with low levels of bird movement.

Differences in bug behavior

When swallow bugs were collected, we noted whether they were inside the nest (requiring removal of the nest), resting on the outside surface of the nest (usually along the bottom and sides), or clustering at the nest entrance in apparent attempts to disperse. Behavior of the bugs

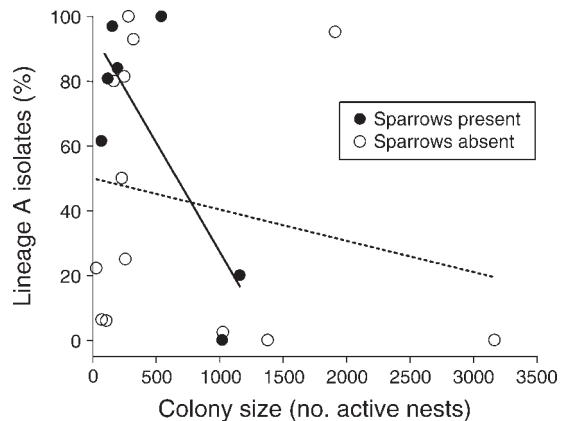


FIG. 3. Percentage of BCRV isolates from bugs that were lineage A in relation to mean Cliff Swallow colony size in years when the site was active (number of active nests) at sites with House Sparrows present (solid circle, solid line) and at sites where there were no active sparrow nests (open circle, dotted line). The percentage of lineage A among the isolates decreased significantly with mean colony size at sites with sparrows ($r = -0.79$, $P = 0.035$, $n = 7$ sites) but not at sites without sparrows ($r = -0.22$, $P = 0.47$, $n = 13$ sites).

infected with the two lineages differed. Across all colonies, among isolates from inside the nests ($n = 108$ isolates), the outside surface of the nests ($n = 204$ isolates), and clustering at the entrances ($n = 43$ isolates), we found that lineage A represented 20.4%, 47.5%, and 58.1%, respectively. Most of the lineage A isolates (84.7%) came from bugs on the exterior of the nest (outside surface or clustering), compared to just over half (59.2%) of lineage B isolates. The distributions of

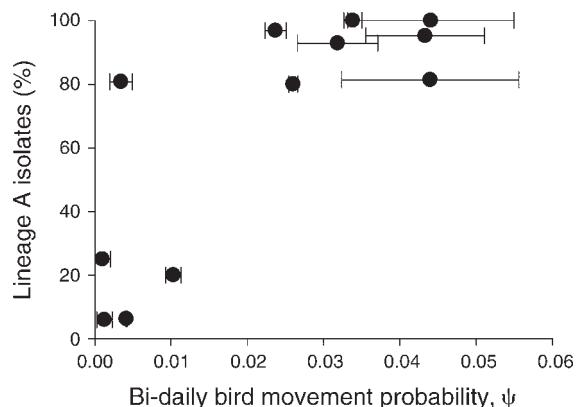


FIG. 4. Percentage of lineage A isolates among the total BCRV isolates from bugs at a Cliff Swallow colony site in relation to the bi-daily (every other day) bird movement probability, ψ (mean \pm SE), into that site from all others in the study area. Yearly movement probabilities into a site, as reported in Brown et al. (2007), were averaged across years at a site for this analysis. The percentage of lineage A among the isolates at a site increased significantly with bird movement probability ($r_s = 0.76$, $P = 0.005$, $n = 12$ sites). The converse held for lineage B.

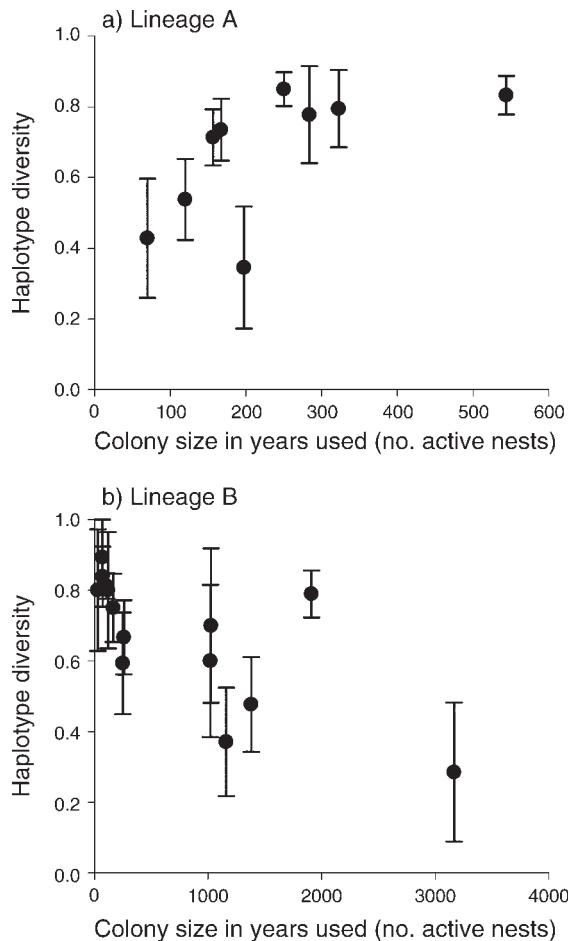


FIG. 5. Haplotype diversity (\pm SE) of BCRV (a) lineage A and (b) lineage B isolates from bugs in relation to mean Cliff Swallow colony size at a site in years when the site was active (number of active nests). Haplotype diversity of lineage A increased significantly with colony size (Spearman rank correlation, $r_s = 0.73$, $P = 0.03$, $n = 9$ sites); that of lineage B decreased significantly with colony size ($r_s = -0.80$, $P = 0.0006$, $n = 14$ sites).

the lineages differed significantly between the positions on the nest ($\chi^2_2 = 27.9$, $P < 0.00001$).

Differences in genetic diversity

Haplotype diversity at a site increased significantly with colony size (mean number of active Cliff Swallow nests in the years a site was used) for lineage A (Fig. 5a) but decreased significantly for lineage B (Fig. 5b). There was no relationship between genetic diversity and substrate type (bridge or culvert). For lineage A, culvert sites exhibited 49.4% unique haplotypes ($n = 154$ haplotypes), compared to 29.4% for bridges ($n = 17$ haplotypes; $\chi^2_1 = 2.5$, $P = 0.12$). For lineage B, culvert sites ($n = 78$ haplotypes) and bridge sites ($n = 108$ haplotypes) each showed 37.0% unique haplotypes. These analyses used only colony sites for which we had more than five isolates identified to lineage.

The presence of House Sparrows at a colony site seemed to affect genetic diversity of lineage A but not of lineage B (Fig. 6). For lineage A, sites with sparrows had significantly more unique haplotypes than at sites with only Cliff Swallows; these percentages were virtually identical for lineage B (Fig. 6).

Differences in cytopathicity and viral RNA concentration

Lineage A exhibited a significantly greater proportion of isolates that were cytopathic on Vero cells (91.7%, $n = 48$) than did lineage B (72.2%, $n = 36$; $\chi^2_1 = 4.3$, $P = 0.04$). Samples of lineage B, however, had significantly higher viral RNA concentration than did samples of lineage A, as measured by ΔC_t values: mean ΔC_t (\pm SE) for lineage A ($n = 89$) was 11.64 (± 0.39), vs. 9.65 (± 0.40) cycles for lineage B ($n = 131$; Wilcoxon test, $Z = 3.19$, $P = 0.001$). The difference of ~ 2.0 in mean ΔC_t values translates to about a fourfold difference in mean viral RNA concentration between samples of the two lineages.

DISCUSSION

Buggy Creek virus is unusual among North American arboviruses in at least four ways: it has been found only at sites with active or inactive Cliff Swallow nests, and thus it is associated only with swallow bug vectors; it persists at high levels in its vector year-round (Brown et al. 2001, 2009a, Strickler 2006, Moore et al. 2007); it is amplified more often in the introduced House Sparrow than in its putative natural vertebrate host, the Cliff Swallow (V. O'Brien and C. Brown, unpublished data); and, as shown here, two distinct co-occurring lineages exist with pronounced ecological differences between them. Because so much is still not known about vector and host dynamics associated with BCRV, we can only

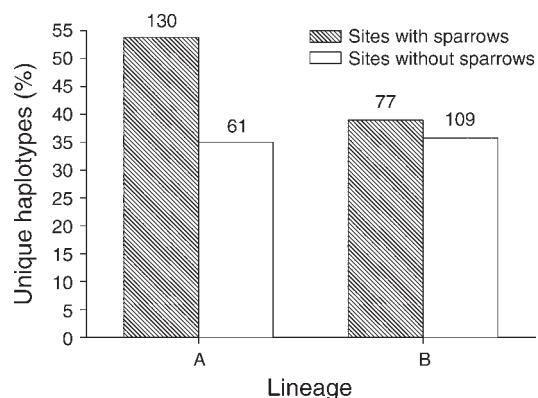


FIG. 6. Percentage of unique haplotypes of BCRV lineages A and B from bugs at sites with House Sparrows (hatched bars) and at sites without House Sparrows (open bars). Sample sizes (total number of isolates) for each lineage and site type are shown above the bars. The percentage of unique haplotypes of lineage A differed significantly between sites with and without sparrows ($\chi^2_1 = 6.3$, $P = 0.01$), but that of lineage B did not ($\chi^2_1 = 0.20$, $P = 0.66$).

speculate as to why these lineages have diverged. Here we focus on two major hypotheses to explain the ecological differences between the lineages.

Divergence in response to vector differences

One possibility is that the two lineages are associated with either different species or subspecies of swallow bugs, in which case the lineages would be diverging in response to differences in the biology of the vector. The lineages do show latitudinal variation in their distribution within the Great Plains, USA, with lineage A the more southerly lineage, found from Oklahoma north to Colorado and Nebraska, and lineage B the more northerly, found from North Dakota south to Colorado and Nebraska (Padhi et al. 2008). This distribution would be consistent with conceivable clinal variation in some aspect of swallow bug biology, with the Nebraska study site representing a region of sympatry between the bug types. The differences we found in the behavior of the bugs infected with the two lineages is also potentially consistent with lineage divergence based on vector differences. However, no taxonomic or phylogeographic studies of *Oeciacus vicarius* have been done, and it has been assumed that swallow bugs consist of a single species throughout the Cliff Swallow's North American range (Usinger 1966). Whether any between- or within-population differences in bugs exist that might reflect virus lineage specialization is unknown.

Divergence in response to House Sparrows as new hosts

The other major hypothesis to explain the divergence of BCRV derives from the close correlation in time between when the two lineages diverged and the arrival of House Sparrows in the study area. Both lineages appear to have diverged from their most recent common ancestor between 60 and 80 years ago, based on molecular substitution data (Padhi et al. 2008), which is about when House Sparrows first arrived in the study area. Sparrows have been present in Nebraska since about 1900 (Robbins 1973) but have been associated with bugs and BCRV since only about 1940, when swallows in our study area started using artificial nesting sites such as bridges and culverts (Brown and Brown 1996) near towns where sparrows occur. Perhaps BCRV lineage B represents essentially the ancestral virus, one that was well adapted to replication and transmission mostly among swallow bugs, not requiring amplification by birds to complete every transmission cycle. Lineage A has possibly diverged to exploit the new host (House Sparrow), which appears to amplify the virus more effectively than does the Cliff Swallow. The molecular data (Padhi et al. 2008) do not reveal which current lineage is closer to the ancestral BCRV, but all of the ecological data presented here are consistent with the two lineages having different transmission cycles and lineage A involving birds to a greater degree.

One lineage associated with birds, the other with bugs

Lineage A peaks in prevalence earlier in the season than lineage B, at a time when large numbers of Cliff Swallows are hatching (Brown and Brown 1996) and the more asynchronous House Sparrows also have nestlings. Because young nestlings of both species (<7 days old) appear to be the ages most commonly infected by BCRV (V. O'Brien and C. Brown, *unpublished data*), a virus lineage requiring a vertebrate amplification cycle should be most prevalent in the vectors when young nestlings are available for replication of virus. In contrast, lineage B was most commonly detected later in the season, peaking in late June and early July at the time in the summer when the bugs are most abundant in the colonies with many instars present (Brown and Brown 2004a) and when nestling Cliff Swallows have started to fledge.

If lineage B is transmitted mostly among bugs, it should be most common at sites with large, stable bug populations. Lineage B was more frequently detected than lineage A at bridge sites, where bug populations are larger and more likely to persist from year to year without sharp population fluctuations, simply because at least some swallows more perennially occupy the large bridges (Brown and Brown 1996). Lineage B was also more likely to occur at the old, established colony sites, where bugs tend to maintain more stable populations, because Cliff Swallows more traditionally use those sites. Lineage A was more prevalent at newer and more erratically occupied sites (where bug populations fluctuate), consistent with it being associated with birds and thus, as we found, more likely than lineage B to be introduced or re-introduced into these ephemeral sites whenever large numbers of immigrant birds arrive there. Cliff Swallows introduce virus to sites principally by carrying infected bugs on their feet and legs (Brown and Brown 2004a, Brown et al. 2008), and adult birds are rarely viremic (O'Brien et al. 2008; V. O'Brien and C. Brown, *unpublished data*).

The increase in genetic diversity of lineage A with colony size also suggests that birds play a role in dispersing this virus lineage. In an earlier study (Brown et al. 2008), we found that haplotype diversity of lineage A increased at sites with greater bird movement into them from elsewhere, probably because sites with heavy bird traffic accumulated haplotypes by virtue of frequent virus introductions by birds. For lineage B, however, there was no such pattern, suggesting that it was less likely to be moved by birds (Brown et al. 2008). The increase in genetic diversity of lineage A at larger colonies reported here is consistent with more frequent virus introductions into larger colonies, perhaps by the higher number of transient birds there (Brown and Brown 2004a, 2005, Brown et al. 2007), which again implicates birds in its spread.

Swallow bugs on the outsides of Cliff Swallow nests (especially those clustering at the entrances) are more likely than those inside a nest to disperse to a new colony

by crawling onto the feet and legs of a passing Cliff Swallow (Brown and Brown 1996, 2004a, 2005). We found lineage A more prevalent in bugs on the outsides of the nests and thus potentially more likely to be dispersed by birds. Lineage B was more likely in bugs inside or behind the nests, where the bugs are less inclined to disperse on birds (C. Brown, *personal observation*).

Because extent of virulence tends to vary directly with the extent of horizontal transmission in many pathogens (Ewald 1994, Day 2002, Stewart et al. 2005), a virus amplified by birds and horizontally transmitted between birds and bugs should be selected to be more virulent than a virus that mostly replicates in bugs or is transmitted nonsystemically (*sensu* Lord and Tabachnick 2002). Consistent with this prediction, the putatively bird-associated lineage A was more cytopathic on vertebrate cells (a measure of virulence) than was lineage B. We note that the greater cytopathicity of lineage A could not be explained by differences in virus titer between samples of the two lineages, because our lineage B samples had higher viral RNA concentrations.

An earlier study found evidence of vertical transmission of BCRV by isolating virus from swallow bug eggs (Brown et al. 2009b). The vertical transmission involved both lineages, although vertical transmission should be more important for lineage B if it is evolving to replicate in bugs without always requiring vertebrate amplification. Sustaining any sort of transmission cycle that does not involve virus amplification by birds would likely require vertical transmission via infected eggs, either transovarially or by infection of the egg casing as it is laid (Tesh 1984, Reisen 1990, White et al. 2005), and/or nonsystemic transmission, either mechanically by many bugs feeding at one place on a bird or nonviremically via circulating blood in heavily parasitized birds (Lord and Tabachnick 2002, Reisen et al. 2007). The increase in lineage B at a time in the season when the largest number of adult bugs and instars are present supports the possibility of bug-to-bug transmission of virus while bugs are feeding.

The presence of House Sparrows seemed to be the best predictor of the relative proportions of the two lineages at a site. Lineage A was more likely than lineage B to be found in bugs at colony sites with active House Sparrow nests than at sites without sparrows, and lineage A was more prevalent at sparrow sites with fewer numbers of Cliff Swallows (i.e., smaller Cliff Swallow colony sizes). This latter result may be because smaller Cliff Swallow colony sizes are generally associated with smaller bug-population sizes (Brown et al. 2001), and less lineage B occurs at sites with smaller, more ephemeral bug populations. Lineage A was also more genetically diverse at sparrow sites than at sites with only Cliff Swallows. The increased diversity at sites with sparrows could reflect more efficient amplification (increased virus replication) in sparrows relative to Cliff Swallows, or greater virus introductions (haplotype

collecting) at a site by sparrows in the same manner as seen for Cliff Swallows (Brown et al. 2008). The latter seems less plausible, because sparrows are relatively sedentary and apparently rarely move long distances between colony sites (Lowther and Cink 1992), in contrast to Cliff Swallows (Brown and Brown 1996). The high competence of House Sparrows as hosts for BCRV (V. O'Brien and C. Brown, *unpublished data*) may be the primary cause of the association between lineage A and sparrows.

Evolution of the lineages

Because the swallow bug is specialized as a Cliff Swallow parasite (Usinger 1966), the association between swallows and bugs is presumably old, and thus Cliff Swallows have probably co-evolved with BCRV since its divergence from its WEEV-related ancestor sometime in the last 1300–1900 years (Weaver et al. 1997). The exclusive association between Cliff Swallows and BCRV (at least until recently) may have led to the evolution of some immunity to the virus by the swallows, especially in light of comparative data showing that colonial species (such as Cliff Swallows) have higher levels of immunity to parasites and pathogens than do more solitary species (Møller et al. 2001). Consequently, this may have promoted the evolution of lineage B as a bug-adapted virus that is less dependent on vertebrate amplification.

On the other hand, data from multiple species suggest that the ancestral life cycle for alphaviruses is probably one of moderate virulence, limited vertical transmission, and the ability to use multiple hosts and (in some cases) vectors (Tesh 1984, Lindsay et al. 1993, Scott et al. 1994, Weaver et al. 1994, 1997, 2004, Brault et al. 1999, Kramer and Fallah 1999, Powers et al. 2000). This suggests that lineage A might be the older one and that lineage B perhaps more recently diverged to become specialized as a bug virus. Lineage B may be a more successful lineage, as some evidence indicates that lineage A has been declining over the last 80 years (perhaps associated with the decline of House Sparrows both continent wide and in Nebraska since 1966; Sauer et al. 2007), and it has been replaced by lineage B at some sites (Padhi et al. 2008). Although we saw no obvious population change during the nine years of our study, we did find, overall, that lineage B is more common in summer. There is little lineage A at sites with only Cliff Swallows, so it may be that the presence of House Sparrows at swallow colonies is essential in maintaining lineage A in the study area.

The divergence of these two virus lineages is largely consistent with previous suggestions that differences in virus strains or subtypes correlate with differences in host or vector competence (Weaver et al. 1994, Powers et al. 2000, Carrington et al. 2005, Weaver 2006). Perhaps the most interesting aspect of BCRV is not lineage A's reliance on birds or even its relationship with House Sparrows, but rather lineage B's ability to persist

and even increase with apparently diminished amplification by birds (e.g., at Cliff Swallow-only sites). Understanding the molecular and immunological basis of the Cliff Swallow's possible resistance to BCRV may help in understanding the selection pressures that caused lineage B to evolve as a predominantly bug virus.

The high competence of invasive House Sparrows as an amplifying host may increase the relative fitness of BCRV lineage A and reduce the selective pressure for lineage B to replicate in the absence of a vertebrate host. The likelihood of this happening may depend in part on how common House Sparrows are in Cliff Swallow colonies and whether they continue to invade new sites. Clearly, BCRV is an unusual arbovirus in many ways, and its two sympatric lineages that use the same vector (and hosts) can provide further opportunities to study virus divergence and the possible emergence of ecologically novel strains.

ACKNOWLEDGMENTS

For field and laboratory assistance, we thank Susan Beckett, Jillian Blackwell, Eric Edwards, Kathryn Gaines, Allison Johnson, Jennifer Klaus, Sarah Knutie, Matt Moore, Cheryl Ormston, Nicholas Panella, Sunita Quick, Sara Robinson, Rajni Sethi, Stephanie Strickler, Karen Winans, and Gudrun Zöller. Kenton Miller and several anonymous reviewers made useful comments on the manuscript. The School of Biological Sciences at the University of Nebraska–Lincoln allowed us to use the Cedar Point Biological Station, and the Union Pacific Railroad and the Robert Clary, Dave Knight, and Loren Soper families allowed us access to land. This work was supported by grants from the National Institutes of Health (R01-AI057569) and the National Science Foundation (IBN-9974733, DEB-0075199, DEB-0514824).

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