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Virus discovery and recent insights into virus diversity in arthropods
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Recent studies on virus discovery have focused mainly on mammalian and avian viruses. Arbovirology with its long tradition of ecologically oriented investigation is now catching up, with important novel insights into the diversity of arthropod-associated viruses. Recent discoveries include taxonomically outlying viruses within the families Flaviviridae, Togaviridae, and Bunyaviridae, and even novel virus families within the order Nidovirales. However, the current focusing of studies on blood-feeding arthropods has restricted the range of arthropod hosts analyzed for viruses so far. Future investigations should include species from other arthropod taxa than ixodida, Culicidae and Phlebotominae in order to shed light on the true diversity of arthropod viruses.

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Introduction
In recent years, the systematic discovery of novel viruses has undergone a renaissance as a field of research [1–3]. Virus discovery has yielded important and in part spectacular new insights into virus diversity. Starting from improved approaches to clone and screen cDNA libraries, the availability of novel amplification-based and sequencing-based techniques has enabled the characterization of viral isolates and even uncultured viruses. Some of the milestones in this field included the untargeted sequencing of the Severe Acute Respiratory Syndrome (SARS) agent [4], discoveries of relevant human respiratory viruses including human metapneumovirus and human coronavirus NL63 [5–7], as well as applications of technology for the clarification of mysterious outbreaks, for instance, one caused by a novel old world arenavirus involving haemorrhagic fever [8].

Out of the necessity to find diagnoses and etiologies in medicine, the field has so far placed a lot of focus on human viruses. Even in veterinary medicine, the paramount discovery of Schmallenberg virus as a novel pathogen in livestock ungulates was driven by clinical necessity, rather than pathogen surveillance [9]. Nevertheless, the growing interest in epidemic preparedness and pathogen ecology has triggered an expansion of fields of application for virus discovery. Efforts to investigate viral animal reservoirs have begun to yield fundamental insight into virus diversity and evolution, exemplified by the clarification of the reservoir of Ebola virus [10], the recent discovery of mammalian viruses co-ancestral with influenza A virus [11], as well as the analysis of the roles of different mammalian orders in paramyxovirus evolution [12]. According to the orientation of pathogen surveillance programmes, those investigations have focused on mammalian and avian viruses. In terms of arthropod-associated viruses, the harvest from virus discovery studies has been thinner [13] but there is now a number of remarkable arthropod virus findings that deserve attention and discussion.

Virus discovery in vertebrates and arthropods — comparison of approaches
It seems unlikely that technical approaches for the same purpose, that is, the discovery of viruses, should differ between fields. However, the huge new interest in virus discovery has made researchers use opportunities within reach. In mammalian and avian viruses, the incentive coming from the medical field has triggered huge investments in technology. Paramount virus discoveries in human medicine, starting from hepatitis B virus in the 1960s through human immunodeficiency virus, hepatitis C virus, and human herpes virus 8 in the early 1990s, have reassured us in our belief that undiscovered viruses would be agents difficult to culture or entirely unculturable. On the contrary, cell culture has been a key technology in the arthropod virus field and it has been long known that unclassified, unknown viruses are routinely cultured from insects and victims of arbovirus infections alike. As a matter of fact, the historical classification of arboviruses in groups a (alphaviruses), b (mainly flaviviruses) and c (other viruses) includes the presumption that a large part of isolates will not be classifiable using traditional methods such as serology. Out of this background, arbovirus researchers have covered continents in their fieldwork and spent decades on collecting viruses.

Consequently, while the investigation of original field samples has come into focus in the mammal and bird virus field, arbovirus researchers have gone back to their
collected cultures, particularly those that remained untypable [14,15]. The collection maintained at the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA) at University of Texas Medical Branch is probably the largest and surely the most prominent of such collections. Other groups have followed similar technical approaches in that they used cell culture as a first-line tool to gain virus isolates, before applying methods aimed at their further characterization [16–19]. There have been a number of important recent studies based on RT-PCR, but these were mainly aimed at the description of novel regions of distribution of known viruses, or close relatives thereof [20–23]. On the contrary, and in spite of demonstrated technical feasibility, there is today not a single exemplary study applying undirected, hypothesis-free deep sequencing on original field samples of arthropods. All studies based on this promising methodology used cell culture isolates as their primary entry point (Figure 1).

Recent results in arthropod virus discovery — viral diversity in arthropods

Flaviviridae (genus Flavivirus)

Viruses that seem to replicate only in mosquito but not in vertebrate cells were first discovered within the genus *Flavivirus*, family *Flaviviridae*. Well-known members of this so-called insect-specific group include cell fusing agent virus, Kamiti River virus, and *Culex flavivirus* (reviewed in [24]). These insect-specific viruses are thought to be maintained in insects only, and to represent ancestral or primordial forms of the vector-borne flaviviruses. Insect-specific flaviviruses have been divided into *Aedes*-associated and *Culex*-associated viruses, respectively. Recent findings of related flaviviruses in mosquito species of other genera [25,26] suggest a much higher diversity of yet-to-be discovered insect-specific flaviviruses. Interestingly, a clade of potentially insect-specific viruses that replicate in mosquito cells but not in vertebrate cells or newborn mice was discovered within the diversity of mosquito-borne viruses. This clade currently has now three members including Nounané virus isolated from *Uranotaenia moshondaensis* mosquitoes originating from the Tai National Park in Côte d’Ivoire [27], Lammi virus isolated from *Aedes cinereus* mosquitoes collected in Finland [28], and Nanay virus, a virus recently isolated from *Culex ocellatus* collected in the Amazonian rainforest near Iquitos, Peru [29]. The diversity of mosquito-associated flaviviruses was further extended by the detection of flavivirus-like sequences integrated within the genomes of *Aedes* mosquitoes [30].
Phylogenetic relationship of prototype arthropod-associated viruses and avian and mammalian viruses. Maximum likelihood analyses of the family Bunyaviridae (a) and the order Nidovirales (b). Arthropod-viruses are shown in red. Associated hosts are indicated by silhouettes to the right.
Togaviridae (genus Alphavirus)
In parallel to the findings within flaviviruses, the first insect-specific alphavirus, named Eilat virus, was discovered in *Anopheles costanti* mosquitoes collected in the Negev desert, Israel [31**]. Phylogenetic analyses placed Eilat virus on a long branch between the Western equine encephalitis serocomplex and *Trocara* virus, a virus isolated from *Aedes serratus* mosquitoes from the Amazon Basin, the prototype of a new antigenic complex [32]. Alphaviruses are divided in two groups, one large group infecting terrestrial mammals and birds via mosquito bites and a second distant related group with two members only infecting fish [33]. The finding of further insect-specific alphaviruses in the future, potentially placed between the aquatic and terrestrial viruses, is very likely.

Bunyaviridae (putative novel genera)
Although the family *Bunyaviridae* is already one of the largest virus families with about 350 members divided into five genera of plant (genus *Tospovirus*) or vertebrate (genus *Orthobunyavirus*, *Phlebovirus*, *Nairovirus*, and *Hantavirus*) pathogenic viruses [34], still many new viruses are being discovered, contributing further to the immense bunyavirus diversity. The case of Schmallenberg virus is only one example why we should continue studying the diversity of arthropod-associated bunyaviruses [9,35]. More examples are provided by severe fever with thrombocytopenia syndrome virus (SFTSV), a novel tick-borne phlebovirus from China isolated from *Haemaphysalis longicornis* ticks, as well as its cousin in North America, the Heartland virus [36–39]. Interestingly, also for the SFTSV and Heartland virus clade, closely related sister taxa named Bhanja virus, Palma virus and Lone Star virus, have now been described in *Haemaphysalis intermedia*, *H. punctata*, *Rhizophyllum decoloratum*, *R. griyn*, *R. pulchellus*, and *Amblyomma americanum*, respectively [40–42].

Moreover, a mosquito-associated phylogenetic outlier virus, termed Gouléako virus, was isolated from different mosquito species (mostly *Culex nebulosus*) collected in the Taï National Park in Côte d’Ivoire [43*]. Gouléako virus did not grow in various primate, bat, bird, rodent, and amphibian cell lines but grew to high titres in mosquito cells suggesting this agent to constitute the first putative insect-specific bunyavirus. Furthermore, a novel clade in phylogenetic sister relationship to orthobunyaviruses and almost equidistant to the established bunyavirus genera has been discovered in mosquitoes originating from Côte d’Ivoire and Uganda [44,45*]. These viruses termed Herbert virus, Taï virus and Kibale virus did also not infect vertebrate cells but replicated well in mosquito cells. Gouléako virus, Herbert virus, Taï virus and Kibale virus may represent clades of insect-specific viruses in co-ancestral relationship to the vertebrate-infecting phlebo- and orthobunyaviruses, respectively. The diversity of the newly described bunyaviruses suggests the existence of further taxa in these clades, as well as the existence of novel clades in basal phylogenetic relationship to other bunyavirus genera (Figure 2).

Nidovirales (novel family Mesoniviridae)
Despite the detection of viruses with distant relationship to established clades and genera, even a novel RNA virus family, named *Mesoniviridae*, was discovered in mosquitoes collected in Côte d’Ivoire and in Vietnam [46**,47**,48]. The monogenic family now contains at least four virus species that seem only to be able to infect insect cells, but not vertebrate cells [49]. The order *Nidovirales* heretofore comprised three families of fish-, bird-, and mammal-infecting viruses, namely the *Coronaviridae* (subfamilies * Coronavirinae* and *Torovirinae*) and the *Arteriviridae*. A third family included crustacean-infecting viruses, the *Roniviridae*. The now expanded nidovirus phylogeny with non-viruses and mesoniviruses branching from deepest tree nodes suggests an origin in arthropods for the whole order *Nidovirales* [46**,49] (Figure 2).

Others (new families, taxa)
Novel insect-specific viruses, collectively termed Negevirus, have been detected in mosquitoes and sandflies captured in Brazil, Peru, the United States, Ivory Coast, Israel, and Indonesia [50*]. These are distantly related to viruses of the genus *Cilevirus* and may represent a novel genus within the same family as cileviruses [50*]. The recently characterized and classified Citrus leprosis virus, a plant virus transmitted by *Brevisalpus* mites, is the type species of the genus *Cilevirus* of an as yet unclassified novel virus family [51]. The wide geographic distribution and the detection in mosquitoes of different genera and sandflies suggest negeviruses to be widely distributed and to potentially infect nonbiting dipteran species widely.

The family *Reoviridae* is the only family of vertebrate-infesting viruses known to occur in a number of non-blood-feeding arthropod species. Two novel viruses were discovered in the three cornered alfalfa hopper (*Spissistilus festinus*) and in the angulate leafhopper (*Acinopterus angulatus*) collected in CA, USA, respectively [52**]. Both viruses were proposed to belong to a new genus of the subfamily *Spinaeovirinae* within the family *Reoviridae*. Other distantly related reoviruses have been isolated from a grass carp (*Ctenopharyngodon idella*) in China [53], from a mud crab (*Scylla serrata*) in China [54], as well as from the winter moth (*Operophtera brumata*) [55].

Conclusions
Recent efforts to discover arthropod viruses have yielded widely divergent taxa that sometimes even define novel families. While much more effort is currently invested in mammal and bird virus detection, the results there seem to have been restricted to the discovery of variants of virus species, sister species to known viruses, and rarely genera.
Reasons for a seemingly wider genetic range of novel arthropod viruses may be technical. Virus culture as employed in most studies should amplify diverse novel viruses— in contrast to molecular biology techniques with their inherent sequence bias. However, studies combining cell culture and next generation sequencing on mammalian and bird viruses still yield a rather limited diversity of novel viruses. Moreover, while a large range of vertebrate cell lines is available, most arthropod-based virus isolation studies relied on only one cell line, namely C6/36 cells from Aedes albopictus. These cells have recently been shown to be deficient in elemental RNA interference (RNAi) components suggesting the cells to be much more susceptible for virus infections [56]. But again, also for mammal and bird viruses there are several cell lines available with defects in their interferon system facilitating virus infection and replication. It is therefore unlikely that differences in our ability to culture viruses could suffice as an explanation for the higher genetic diversity in novel virus discoveries in insects, as opposed to vertebrates. Notably, while undirected studies based on next generation sequencing are not available for arthropods, they have indeed been conducted intensely for mammals and birds. Even if some of those studies mentioned sequence reads that could neither be ascribed to the host genome or transcriptome, nor to known viral families, the absence of discoveries of highly divergent novel taxa including novel families in mammals and birds is striking. The obvious and most likely explanation is sampling bias. Our current coverage of host diversity is highly limited. In mammalian and bird virus discovery, huge efforts have been spent historically on human hosts, as well as companion and livestock animals [12]. Triggered by findings of important zoonotic agents such as SARS-CoV, Hanta-, and influenza viruses, the array of mammal and bird species of interest has been extended to wild rodents, bats, and migratory birds [1,2]. Many of the more important recent findings of novel vertebrate viruses have been made there, confirming conjectures that taxa with huge social group sizes, population turnover, and migratory behaviour favour the emergence and maintenance of viruses.

On the contrary, no such considerations have been made so far in the design of sampling for arthropod virus discovery. Sampling has been determined (and limited) by our interest in blood-feeding arthropods, such as mosquitoes, sandflies, and ticks. These are not closely related, with an evolutionary distance of ca. 706 million years [57], in contrast to birds and mammals that separated ca. 160 mya [58]. If we accept virus and host diversity to be roughly correlated, we have to admit that we are probably overlooking the largest part of existing arthropod virus diversity. Arthropods are the largest animal phylum including the class of insects which comprises more than half of all described species [59]. Critically, it has to be considered that viruses may not only be acquired from insects via the blood-borne route but also by the ingestion of infectious diet. Arthropods are a major dietary component for a large range of small vertebrates, and we would be well advised to consider virus discovery studies on those taxa that are an attractive prey for small mammals and birds— such as moths, butterflies, caterpillars, bugs, or spiders.

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