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Tibet Orbivirus, a Novel Orbivirus Species Isolated from Anopheles maculatus Mosquitoes in Tibet, China

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Abstract

Background: The genus Orbivirus includes a number of important pathogenic viruses, including Bluetongue virus (BTV), African horse sickness virus (AHSV), and Epizootic hemorrhagic disease virus (EHDV). In this study we describe the isolation and characterization of an Orbivirus strain isolated from Anopheles maculatus mosquitoes collected in Tibet, China.

Methods and Results: Initial viral screening identified a viral strain (XZ0906) that caused significant cytopathic effect (CPE) in BHK-21 cells, including rounding, cell rupture, and floating. Although CPE was not observed in insect cells (C6/36), these cells supported viral replication. Polyacrylamide gel analysis revealed a genome consisting of 10 segments of double-stranded RNA (dsRNA), with a distribution pattern of 3-3-3-1. 454 high throughput sequencing of culture supernatant was used for viral identification. Complete genome sequencing was performed by Sanger sequencing in combination with 5' RACE and 3'-RACE. Sequence analysis demonstrated that all 5' and 3' untranslated regions (UTRs) for each of the 10 genome segments contained a series of six highly conserved nucleotides. In addition, homology analysis and phylogenetic analysis based on amino acid sequence was completed, and all results show that virus XZ0906 was not a member of any known species or serotype of Orbivirus, indicating it to be a new species within the genus Orbivirus.

Conclusions: The isolated Orbivirus strain was designated Tibet Orbivirus, TIBOV to denote the location from which it was isolated. TIBOV is a novel orbivirus species which is isolated from Anopheles maculatus mosquitoes collected in Tibet, China.

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Introduction

There are currently 22 confirmed species of the genus Orbivirus in the family Reoviridae [1]. This genus includes a number of important pathogenic viruses, including Bluetongue virus (BTV), African horse sickness virus (AHSV), and Epizootic hemorrhagic disease virus (EHDV) [1,2], which are spread primarily through insect vectors, such as Culicoides midges, ticks, mosquitoes, and phlebotomine flies [1,3-6].

Orbiviruses contain a multi-segmented, double-stranded RNA genome, consisting of 10 segments (Seg1–Seg10) of various length, which are identified according to their molecular weight [7]. Partial nucleotide sequences for each of the gene segments for many of the Orbiviruses have been published, along with complete genome sequences of some species [3,5,8–10], allowing for detailed classification and phylogenetic analysis of Orbiviruses.

This study describes a viral strain (XZ0906) isolated from Anopheles maculatus specimens collected in Tibet, China. All the results of initial viral screening showed a difference between XZ0906 and Yunnan Orbivirus (YUOV), an orbivirus also isolated from China. After whole genome sequencing, amino acid homology and molecular phylogenetic analysis, XZ0906, which is designated as Tibet Orbivirus (TIBOV), is identified as a novel species of the genus Orbivirus.

Materials and Methods

1. Cell culture
Aedes albopictus C6/36 cells and BHK-21 (Baby hamster kidney) cells (ATCC) were used in this study [11], and both cell lines were kept in our laboratory. C6/36 cells were maintained in medium with 45% RPMI 1640 and 45% DMEM (Invitrogen) supplemented with 10% inactivative fetal bovine serum (FBS, Invitrogen) and 100 U/mL penicillin and streptomycin. Cells were propagated and maintained at 28°C [11–13]. BHK-21 cells were grown in minimal essential medium with Eagle's balanced salt solution supplemented with 10% FBS (Invitrogen), 2 mM glutamine, 0.12% NaHCO3, and 100 U/mL penicillin and streptomycin.
Table 1. Primers used in this study.

| Primer | Sequence (5’-3’) | Position | Orientation |
|--------|------------------|----------|-------------|
| 6-1-1F | GTAAATCATATAATGTCG | 1–18 | Sense |
| 6-1-1R | TACGAGCAATCTCCCCAAG | 826–843 | Antisense |
| 6-1-2F | TGAAGAGGAGGGGCTGAG | 679–697 | Sense |
| 6-1-2R | TAGACCTCTTGTTTTGGT | 1531–1548 | Antisense |
| 6-1-3F | AGTCGAAAAGAAGTTTGGT | 1385–1402 | Sense |
| 6-1-3R | CGACGTAAATATACGCTT | 2310–2327 | Antisense |
| 6-1-4F | ATTTAGCATGATAGCACAG | 2152–2171 | Sense |
| 6-1-4R | GAGAAATGCCCGGTGTT | 3064–3081 | Antisense |
| 6-1-5F | ATGGGACCCCCATCATAA | 2874–2891 | Sense |
| 6-1-5R | CGTCTCCTCCCTGCACAA | 3786–3803 | Antisense |
| 6-1-6F | CTGAAATAATGGATCCTGTTGA | 3019–3040 | Sense |
| 6-1-6R | GTAAATGTAGATAGCGCC | 3926–3950 | Antisense |
| 6-2-1F | GTAAACCTGAGCTTGGAAGACCTT | 1–25 | Sense |
| 6-2-1R | CGACTCCCTCTCTGAAAT | 940–957 | Antisense |
| 6-2-2F | ATTTGGGAATGTTGAGT | 760–777 | Sense |
| 6-2-2R | TTCATACGTTGTTGTAAG | 1549–1566 | Antisense |
| 6-2-3F | TTAATAGTTGATGTTGCACTT | 1428–1447 | Sense |
| 6-2-3R | CATCCTTACCTTGCACGG | 2270–2287 | Antisense |
| 6-2-4F | GGGCATACGGCGGAGAAT | 2021–2038 | Sense |
| 6-2-4R | GTAAGTAAATCTGCTGTGATC | 2864–2888 | Antisense |
| 6-3-1F | GTAAATTTCTGGCCGATGCTGTA | 1–25 | Sense |
| 6-3-1R | ACCGGAGTGGTATGATGT | 824–841 | Antisense |
| 6-3-2F | GCTCGGACCCACTTTACC | 637–654 | Sense |
| 6-3-2R | TGCTGCCACAAGCATCAG | 1515–1532 | Antisense |
| 6-3-3F | GTAGTCTGGCAATCTCGT | 2248–2265 | Sense |
| 6-3-3R | TATAATGGATGGGCTGTC | 1356–1373 | Antisense |
| 6-3-4F | GTAAGTGTATTCCCGTTGCAGTCGG | 2745–2769 | Antisense |
| 6-3-4R | TATTGGAGCGTGAAGCAT | 2056–2073 | Sense |

BHK-21 cells were propagated and maintained at 37°C under a 5% CO2 atmosphere [11–13].

2. Viral isolation

Mosquito samples were collected in Medog County (altitude 1000 m) in the Nyingchi area of Tibet, China during the summer of 2009, and transported to the laboratory in liquid nitrogen containers, following morphological classification and species identification on-site. All specimens were homogenized and centrifuged at 12000 x g for 30 min at 4°C. To isolate the virus, 150 μL of supernatant was then added to monolayers of both C6/36 and BHK-21 cells, and cultured at 28 and 37°C, respectively, in a 5% CO2 incubator. Cells were monitored at 24-h intervals to identify cytopathic effects (CPE) associated with infection [11–13].

3. dsRNA-polyacrylamide gel electrophoresis

Viral RNA was isolated as described previously, and analyzed by polyacrylamide gel electrophoresis [13].

4. Preparation of viral DNA and RNA and 454 sequencing

Viral DNA was extracted from 200-μL aliquots of virus-infected BHK-21 cell culture supernatants using a QIAamp DNA Blood Mini Kit (Qiagen). Viral RNA was extracted from 140-μL aliquots of virus-infected BHK-21 cell culture supernatant using a QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer’s instructions. cDNA was made with a Ready-To-Go kit (GE Healthcare) using random hexanucleotide primers. Samples were then amplified as described previously [14,15]. Amplification products were pooled, adaptor-ligated, and sequenced at the Washington University Genome Sequencing Center on the 454 GS-FLX platform (454 Life Sciences, Branford, CT).

Because the nucleic acids used for sequencing contained a mixture of host cell DNA and viral RNA, sequencing reads were filtered using the custom informatics pipeline VirusHunter [16] to identify viral sequences. Sequences identified as most similar to viruses in the genus Orbivirus, as well as those that had no significant hit to any sequence in the GenBank database, were assembled with Newbler (454 Life Sciences) using the default parameters. Sequences were trimmed to remove primer sequences prior to data analysis and assembly.
| Genus                      | Species                        | Abbreviation | Strain/Serotype       | GenBank accession no. |
|---------------------------|--------------------------------|--------------|-----------------------|-----------------------|
| Genus Orbivirus           | African horsesickness virus    | AHSV-1       | HS29-62/serotype1     | FJ183364              |
|                           | AHSV-2                         | HS02-07/     | FJ196584              |
|                           | AHSV-4                         | HS32-62/     | JQ796724              |
|                           | AHSV-9                         | E41-02(Or)/  | U94887                |
|                           | Bluetongue virus               | BTV-1        | S297-1/serotype1      | JN848759              |
|                           | Bluetongue virus               | BTV-1A       | Australia             | P20608                |
|                           | Bluetongue virus               | BTV-2        | BTV-2IT(L)/serotype2  | JN255862              |
|                           | Bluetongue virus               | BTV-4        | BTV-4IT(L)/serotype4  | JN255882              |
|                           | Bluetongue virus               | BTV-6        | USA2006-01/serotype6  | GQ506536              |
|                           | Bluetongue virus               | BTV-9        | BTV-9IT(L)/serotype9  | JN255902              |
|                           | Bluetongue virus               | BTV-12       | BTV12-PT2003/serotype12 | GU390658              |
|                           | Changuinola virus              | CGLV         | BeAr478620            | HQ397615              |
|                           | Corriparta virus               | CORV         | CSIRO1740             | HQ397617              |
|                           | Epizootic hemorrhagic disease virus | EHDV-1   | New Jersey/serotype1  | NC_013396              |
|                           | Epizootic hemorrhagic disease virus | EHDV-2 | Ibaraki/serotype2     | AM745077              |
|                           | Epizootic hemorrhagic disease virus | EHDV-2 | Alberta/serotype2     | AM744997              |
|                           | Epizootic hemorrhagic disease virus | EHDV-6 | 318/serotype6         | AM745067              |
|                           | Equine encephalosis virus      | EEV          | HS103-06              | FJ183384              |
|                           | Eubenangee virus               | EUBV         | AUS1963/01            | JQ070376              |
|                           | Great Island virus             | GIV          | CanAr-42              | ADMM88592             |
|                           | Broadhaven virus               | BRDV         | BRDV                  | NA                    |
|                           | Kemerovo virus                 | KEMV         | EgAn 1169-61          | ADMM8609              |
|                           | Lipovnik virus                 | LIPV         | CzArLip-91            | ADMM8603              |
|                           | Tribec virus                   | TRBV         | TRBV                  | ADMM8606              |
|                           | Itupiranga virus               | ITUV         | BeAr121086            | HQ397639              |
|                           | Maticare virus                 | MATV         | MARU21343             | HQ397640              |
|                           | Orungo virus                   | ORUV         | IBH11306-84           | H397641               |
|                           | Palyam virus                   | PALV         | Chuzan                | BAA76549              |
|                           | St Croix River virus           | SCRv         | SCRv                  | AAG34363              |
|                           | Umatilla virus                 | UMAV         | USA1969/01            | AEE98368              |
|                           | Stretch Lagoon virus           | SLOV         | K49460                | ACH91290              |
|                           | Wallal virus                   | WALV         | Ch12048               | NA                    |
|                           | Warrego virus                  | WARV         | VS080                 | ABM92924              |
|                           | Warrego virus                  | WARV         | Ch9935                | ABM99690              |
|                           | Wongorr virus                  | WGRV         | CSIROS1               | H397668               |
|                           | Wongorr virus                  | WGRV         | mrm13443              | NA                    |
|                           | Wongorr virus                  | WGRV         | Paroo-River           | NA                    |
|                           | Yunnan orbivirus               | YUOV         | YOV-77-2              | YP443925              |
|                           | Middle point orbivirus         | MPOV         | DPP4440               | ABU95014              |
| Genus          | Species                          | Abbreviation | Strain/Serotype | GenBank accession no. |
|---------------|----------------------------------|--------------|-----------------|-----------------------|
|               |                                  |              | VP1(RdRp)       | T 2                   |
| Genus Phytoreovirus | Rice dwarf virus              | RDV-A        | A               | BAA14222              |
|               | Rice dwarf virus                | RDV-Ch       | Chinese         | AAB18743              |
|               | Rice dwarf virus                | RDV-H        | H               | BAA01074              |
| Genus Rotavirus    | Rotavirus A (Bovine rotavirus A) | BoRV-A/UK   | UK WT BRV4A     | CAA39085              |
|               | Rotavirus A (Bovine rotavirus A) | SIRV-A/SA11  | Simian          | AACS6684              |
|               | Rotavirus C (Porcine rotavirus C) | PoRV-C/Co    | Co              | AAB00801              |
| Genus Seadornavirus | Banna virus                   | BAV          | BAV-Ch          | AAFF77631             |
|               | Kadipiro virus                  | KDV          | JKT-7015        | AAFF78848             |
|               | Liao ning virus                 | LNV          | LNSV-NE9731     | AAQ83562              |
| Genus Cardoreovirus | Eriocheir sinensis reovirus    | ESRV         | 905             | AAT111887             |
| Genus Mimoreovirus | Micromonas pusilla reovirus   | MPRV         | MPRV            | AAZ94041              |
| Genus Aquareovirus | Aquareovirus A (Chum salmon reovirus) | CSRV       | CSRV            | AAL31497              |
|               | Aquareovirus A (Striped bass reovirus) | SBRV     | SBRV            | AAM93410              |
|               | Aquareovirus C (Grass carp reovirus) | GCRV      | GCRV            | AAG10436              |
|               | Aquareovirus C (Golden shiner reovirus) | GSRV     | GSRV            | AAM92745              |
|               | Aquareovirus G (Golden ide reovirus) | GIRV     | GIRV            | AAM93415              |
| Genus Cypovirus    | Dendrhyus punctatus cytoplasmic polyhedrosis virus-1 | DsCPV-1 | DsCPV-1         | AAN46860              |
|               | Lymnantria dispar cytoplasmic polyhedrosis virus-14 | LdCPV-14 | LdCPV-14        | AAK73087              |
| Genus Coltivirus | Colorado tick fever virus       | CTVF         | Florio          | AAK00595              |
|               | Eyach virus                     | EYAV         | Fr578           | AAM18342              |
| Genus Dinovernavirus | Aedes pseudoscutellaris reovirus | APRV    | APRV            | AAZ94068              |
| Genus Fijivirus | Nilaparvata lugens reovirus     | NLRV-Iz      | Izumo           | BAA08542              |
| Genus Mycoreovirus | Mycoreovirus 1 (Cryphonectria parasitica reovirus) | CpMYRV-1   | 9B21            | AAP45577              |
|               | Mycoreovirus 3 (Rosellinia RnMYRV-3 anti-rot virus) | RArV      | RArV            | BAC98431              |
| Genus Orthoreovirus | Mammalian orthoreovirus MRV-1  | Lang         | Lang            | AAA47234              |
|               | Mammalian orthoreovirus MRV-2  | Jones        | Jones           | AAA47245              |
|               | Mammalian orthoreovirus MRV-3  | Dearing      | Dearing         | AAA47255              |
|               | Mammalian orthoreovirus MRV-4  | Ndele        | Ndele           | AAL36027              |
| Genus Oryzavirus | Rice ragged stunt virus         | RRSV-Th      | Thai            | AAC36456              |

**Note:** NA, Not available.

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5. Complete genome sequencing including 5'- and 3'-untranslated regions

Reverse-transcription polymerase chain reaction (RT-PCR) was performed to fill in gaps between viral gene sequences obtained with 454 sequencing using contig-specific primers. Total viral RNA was extracted as described in Step 4, cDNA was generated by reverse transcription, and used as a template for complete genome amplification. Next, a set of specific primers was designed to amplify each segment of the viral genome and the amplification products were sequenced using the Sanger method (Table 1). 5'-RACE and 3'-RACE systems (Rapid Amplification of cDNA Ends), Version 2.0 (Invitrogen) were used to amplify the 5'- and 3'-UTRs from each of the 10 segments, respectively. 5'-RACE was performed according to the manufacturer’s instructions. For 3'-RACE, a PolyA tail was first added to RNA using a PolyA polymerase. 3'-UTR sequences were then generated by RT-PCR using sequence-specific and oligo-dT-adapter primers. Sequence assembly was performed resulting in a complete viral genome.
6. Molecular detection of viral genes in cell culture

Viral replication was detected in infected C6/36 and BHK21 cells using RT-PCR for specific regions for TIBOV segment 1 and segment 2. Total RNA was extracted from cell culture supernatants as described in Step 4. cDNA was then generated by reverse transcription, and used as a template for RT-PCR. Gene amplification was performed using primers 6-1-5R and 6-1-5F (primers for Seg1), 6-2-2R and 6-2-2F (primers for Seg2), etc.

Figure 4. Contigs assembled from 454 sequencing reads compared with BTV. Blue bars represent RNA segments from the BTV reference genome; red bars represent assembled viral contigs. Contig lengths and coverage are shown below each of the respective contigs. doi:10.1371/journal.pone.0088738.g004

Table 3. Lengths of the coding and untranslated regions of each of the 10 genomic segments of virus XZ0906.

| Segment | Length (bp) | Protein (aa) | 5' UTR | Terminal sequence | 3' UTR |
|---------|-------------|--------------|--------|-------------------|--------|
|         | Length (bp) |              |        |                   |        |
| S1      | 3950        | 1304         | 11     | 5'-GUAAAAUC--     | 24     | --ACACUUAC-3' |
| S2      | 2888        | 946          | 13     | 5'-GUAAAAAC--     | 34     | --AAACUUAC-3' |
| S3      | 2769        | 899          | 17     | 5'-GUAAAAAUU--    | 52     | --ACACUUAC-3' |
| S4      | 1978        | 643          | 8      | 5'-GUAAAAAAC--    | 38     | --ACACUUAC-3' |
| S5      | 1775        | 554          | 31     | 5'-GUAAAAAA--     | 79     | --ACACUUAC-3' |
| S6      | 1636        | 526          | 26     | 5'-GUAAAAAA--     | 29     | --AAACUUAC-3' |
| S7      | 1165        | 349          | 17     | 5'-GUAAAAAUU--    | 98     | --ACACUUAC-3' |
| S8      | 1142        | 359          | 20     | 5'-GUAAAAAAA--    | 42     | --AAACUUAC-3' |
| S9      | 1100        | 346          | 14     | 5'-GUAAAAAA--     | 45     | --AAACUUAC-3' |
| S10     | 832         | 234          | 21     | 5'-GUAAAAAA--     | 106    | --CAACUUAC-3' |

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Table 4. Comparison of each segment between virus XZ0906 and other Orbiviruses in nucleotide numbers and amino acid identities.

| Segment | AHHSV-4 | BTV-6 | EHDV-6 | PALV | SCRV | YUOV |
|---------|---------|-------|--------|------|------|------|
|          | nt      | aa(%) | nt     | aa(%) | nt   | aa(%) | nt   | aa(%) |
| S1      | 3965    | 1305(59.8) | 3944  | 1302(71.9) | 3942  | 1302(72.9) | 3930  | 1295(59.2) | 4089  | 1345(35.3) | 3993  | 1315(47.8) |
| S2      | 3229    | 1060(9.9)  | 2922  | 955(28.8)  | 2971  | 972(24.6)  | 3055  | 1002(15.6) | 2747  | 890(16.7)  | 2900  | 940(16.3)  |
| S3      | 2792    | 905(58.5)  | 2772  | 901(75.9)  | 2768  | 899(75.8)  | 2774  | 904(58.0)  | 2024  | 654(13.1)  | 2688  | 873(8.8)   |
| S4      | 1978    | 642(50.5)  | 1981  | 644(65.5)  | 1983  | 644(64.4)  | 1967  | 640(48.7)  | 2017  | 643(34.2)  | 1993  | 645(40.7)  |
| S5      | 1748    | 548(27.6)  | 1769  | 552(38.5)  | 1803  | 551(41.6)  | 1764  | 545(25.3)  | 1664  | 517(8.8)   | 1957  | 574(20.1)  |
| S6      | 1566    | 505(43.6)  | 1637  | 526(58.4)  | 1641  | 527(61.4)  | 1610  | 521(43.3)  | 1657  | 517(8.6)   | 1683  | 535(31.6)  |
| S7      | 1167    | 349(56.7)  | 1157  | 349(69.1)  | 1162  | 349(69.3)  | 1151  | 348(54.1)  | 1463  | 462(8.8)   | 1504  | 435(17.2)  |
| S8      | 1166    | 365(36.3)  | 1125  | 354(47.3)  | 1186  | 373(44.5)  | 1059  | 333(40.3)  | 1256  | 379(9.9)   | 1191  | 355(16.4)  |
| S9      | 1160    | 366(32.9)  | 1046  | 328(52.4)  | 1140  | 359(46.5)  | 877   | 272(43.3)  | 764   | 232(35.3)  | 1082  | 338(39.8)  |
| S10     | 756     | 217(30.7)  | 822   | 229(53.9)  | 810   | 228(51.0)  | 728   | 211(28.0)  | 764   | 224(17.4)  | 825   | 253(14.9)  |

Note: As the T2 protein of Orbiviruses had important functions in virus protein/RNA structure and assembly, amino acid homology analysis for the T2 protein of TIBOV (T2 = VP3) compared to the T2 proteins of the above mentioned orbiviruses is presented: AHHSV-4(T2 = VP3):58.5%; BTV-6(T2 = VP3):75.9%; EHDV-6(T2 = VP3):75.8%; PALV(T2 = VP3):58.0%; SCRV(T2 = VP2):22.9%; YUOV(T2 = VP2):37.6%.

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detailed sequence information for all primer sequences is shown in Table 1. PCR was performed under the following conditions: one cycle of denaturation at 95°C for 5 min, 35 cycles of 95°C for 1 min (denaturation), 52°C for 1 min (annealing), and 72°C for 1 min (extension), followed by a final extension at 72°C for 10 min. Amplification products were analyzed by gel electrophoresis on a 1% agarose gel.

7. Nucleotide and amino acid sequence analysis

Sequences were identified by BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple sequence alignments were performed using the Clustal X2 software. Phylogenetic analysis of amino acid sequences for each Orbivirus gene segment were performed using the MEGA 5.04 software package (www.megasoftware.net). Amino acid sequences were analyzed using PredictProtein (http://www.predictprotein.org/). The background information for all virus strains used in this study is shown in Table 2.

Results

1. Isolation of viral strains

A. maculatus mosquitoes collected from Tibet, China were homogenized, and the supernatant added to monolayers of C6/36 and BHK-21 cells. Severe CPE was observed in BHK-21 cells three days after inoculation with mosquito lysate XZ0906, characterized by cell rounding, lysis, and floating cells (Figure 1). However, no obvious pathological changes were seen in C6/36 cells cultured with the same mosquito lysate for five days, or after three consecutive passages. Despite the lack of CPE in C6/36 cells, Orbivirus Seg1 and Seg2 could be detected by RT-PCR in the supernatant of third-generation C6/36 cultures (Figure 2), indicating that virus XZ0906 could replicate in C6/36 cells.

2. Identification of a segmented dsRNA genome

Viral RNA was harvested from the culture supernatant of infected BHK-21 cells, and analyzed by polyacrylamide gel electrophoresis (PAGE), revealing a genome consisting of 10 dsRNA segments, whose migration pattern was 3-3-3-1 (Figure 3). Within this pattern Seg2 migrated to the same region as Seg3; Seg5 and Seg6 were also difficult to distinguish, indicating that these segments had similar molecular weights. Segments 7, 8, and 9 were also similar in terms of molecular weights, but were easily distinguished from Seg10.

3. Preliminary identification of virus XZ0906 using 454 sequencing

Following random PCR amplification, samples were pooled (with barcodes) along with other samples, and sequenced using the Roche/454 FLX Titanium platform, producing a total of 24,929 reads. Sequence data were analyzed using the customized data analysis pipeline VirusHunter [16], identifying 85 unique reads which exhibited 28.1–84.9% sequence identity to viruses in the genus Orbivirus.

All individual reads with detectable similarity to Orbivirus, as well as those sharing no detectable sequence similarity with any sequence in the GenBank database, were used as inputs and assembled into contigs using the Newbler assembler. Twenty-one contigs were assembled, of 138–1342 bp in length, with the greatest similarity to BTV at a coverage depth of 1.4–20.9-fold (Figure 4). Almost-complete RNA sequences were obtained for segments 7, 8, 10. Segments 1, 3, 4, 6 and 9 were represented by two to five contigs; a single contig was identified for segments 2 and 5.

4. Sequencing and analysis of virus XZ0906 and other Orbiviruses

RT-PCR amplification was used to close the gaps between contigs for each of the 10 segments. Primer walking, together with 5’- and 3’-RACE, were used to sequence the 5’- and 3’-ends of each segment. Finally, Sanger sequencing was employed to confirm sequences using primers newly designed for each of the 10 RNA segments (Table 1); complete sequences for this virus XZ0906 have been deposited in GenBank under accession number(genome segments KF746187 to KF746196).

Sequence analysis identified a stretch of six highly conserved nucleotides present at the ends of the 5’- and 3’-UTRs (5’-
GUAAA and ACUUAC-3', respectively) for each of 10 gene segments (Table 3). Significant differences were observed in both the nucleotide and amino acid sequences of virus XZ0906 relative to other members of the genus Orbivirus (Table 4). The VP1 protein (RNA-dependent RNA polymerase, RdRp), encoded by Seg1, shared 35.3% (SCRV)-72.9% (EHDV-6) identity at the amino acid level to the six selected Orbiviruses. Protein T2, encoded by Seg3 of XZ0906, shared 22.9% (SCRV) to 75.9% (BTV-6) identity (Table 4).

5. Phylogenetic analysis and classification of virus XZ0906

5.1. Phylogenetic analysis of virus XZ0906 based on VP1 amino acid sequences. To better understand the taxonomic classification of virus XZ0906, the amino acid sequences of 37 VP1 proteins (Table 2) covering 14 genera within the family Reoviridae were obtained from GenBank, and used to construct a phylogenetic tree. These 37 virus strains (including different species and different serotype of one species) readily clustered into 14 evolutionary branches, with virus XZ0906 clustering within the genus Orbivirus branch (Figure 5(A)). To further establish the taxonomic classification of virus XZ0906, VP1 amino acid sequences from 28 known Orbivirus strains were used to construct a phylogenetic tree specific to this genus (Table 2). This analysis shows that virus XZ0906 forms a unique phylogenetic branch independent of any known Orbivirus species (Figure 5(B)).

5.2. Phylogenetic analysis based on the T2 protein amino acid sequence. The amino acid sequence of the T2 protein is an important marker used to classify species within the genus Orbivirus. T2 amino acid sequences from 29 known Orbivirus strains, along with the equivalent region from virus XZ0906, were selected to construct a phylogenetic tree. This analysis showed that virus XZ0906 is independent of any known Orbivirus species (Figure 5(C)). From these results, we determined virus XZ0906 to represent a novel species within genus Orbivirus. This novel species was given the name Tibet Orbivirus, TIBOV to reflect the location from which it was isolated.
Discussion

According to the 9th meeting report of the International Committee on the Taxonomy of Viruses (ICTV), the Reoviridae family consists of 15 genera: Orbivirus, Rotavirus, Saugavirinae, Phytoreovirus, Cardiovirus, Minovirus, Aquareovirus, Calicivirus, Cytopivirus, Dinocevirus, Figivirus, Iridovirus, Myxocovirus Orthoreovirus, and Orzyavirus [1]. All Reoviridae genomes consist of multi-segmented dsRNA; for example, the genome of Saugavirinae, Rotavirus, and Orbivirus contain 12, 11, and 10 dsRNA segments, respectively [2,10,17,18]. Here we describe a novel orbivirus species isolated from mosquitoes collected in Tibet. This virus has many features characteristic of orbiviruses.

UTRs were detected at both the 5’ and 3’-ends of all 10 TIBOV gene segments. The lengths of these UTRs were highly variable; however, all 3’-UTRs contained a stretch of six highly conserved nucleotides at the end, which is a defining molecular characteristic used in the identification of Orbiviruses [8]. For BTV, AHSV, PALV, and Equine encephalitis virus (EEV), this stretch of six conserved nucleotides is readily detected in the 3’-UTRs of each gene segment [1,4]; however, no such sequences are found at their corresponding 5’-ends. Among the 10 gene segments in Yunnan virus (YUOV), a recently identified Orbivirus isolated from mosquitoes in Yunnan, China, nine (Seg2–Seg10) contained a conserved seven-nucleotide sequence at the 5’-UTR end, but only three conserved nucleotide sequences at the 3’-end [4]. Among the 10 gene segments of Tibet Orbivirus (TIBOV), six conserved nucleotide sequences were detected in both end of the 5’- and 3’-UTRs (5’-GUAAAA and ACUUAC-3’, respectively); these sequences were distinct from those in any other Orbivirus species.

The Orbivirus RNA-dependent RNA polymerases (RdRp), which is encoded by the Segl gene [VP1], is an important maker for species identification [4,8]. The VP1 protein sequence similarities of TIBOV to those of other Orbivirus species were 35.3–72.9% (Table 4), indicating that TIBOV constituted a novel member of the genus Orbivirus. In addition, the T2 protein of Orbivirus is used to classify serotypes within the genus, with a threshold >91% identity at the amino acid level [4,19,20]. Such as Middle Point orbivirus (MPOV), which is isolated from YUOV, and highlights the level of genetic diversity within Orbiviruses in China.

Orbiviruses can be transmitted by ticks or other hematophagous insect-vectors, including Culicoides, mosquitoes, and sand flies [1,9]. The phylogenetic analyses (Figure 5(C)) indicated that TIBOV, isolated from A. maculatus, clustered with Orbiviruses which are transmitted primarily by Culicoides [1,4,9,10], such as BTV, EHDV, and AHSV. TIBOV is more distantly related to Orbiviruses which are isolated from mosquito specimens, such as YUOV [3], Peruvian horse sickness virus (PHSV) [9], Umatilla virus (UMAV) [10], and Stretch Lagoon Orbivirus (SLOV) [9,10]. Further study is necessary to determine if TIBOV is transmitted exclusively through A. maculatus, or can be spread by other blood- sucking insects.

TIBOV was isolated from A. maculatus specimens collected at a pigsty in rural Tibet. It is currently unknown whether TIBOV can infect either humans or animals. In order to determine whether this virus poses a risk of public health, serological studies to define potential human and animal exposures to TIBOV are needed.

Author Contributions

Conceived and designed the experiments: GL ML. Performed the experiments: ML YZ GZ SF. Wrote the paper: GL YZ ML GZ DW. Critical revision of the manuscript: GL DW.

References

1. Attoui H, Mertens PPC, Becq J, Belaganahalli S, Bergoin M, et al. (2011) Ninth Report of the International Committee on Taxonomy of Viruses. In: Andrew MQ King, Michael J Adams, Eric B Carstens, Elliot J Lefkowitz, editors. Family: Reoviridae. London: Elsevier/Academic Press. pp. 541–603.

2. Mertens PPC (1999) In Encyclopedia of Virology, 2nd edn. In A. Granoff&R.G., editors. Orbiviruses and caliciviruses-general features. Webster: London. Academic Press. pp. 1043–1061.

3. Attoui H, Jaafar FM, Belhouachet M, Al-Ahmad F, Taso O, et al. (2005) Yunnan orbivirus, a new Orbivirus species isolated from Culex tritaeniorhynchus mosquitoes in China. J Gen Virol 86: 3409–3417.

4. Moss SR, Jones LD, Nuttall PA (1992) Comparison of the major structural core proteins of tick-borne and Culicoides-borne Orbiviruses. J Gen Virol 73: 2305–2390.

5. Attoui H, Stirling JM, Munroehoh UG, Billor F, Brookes SM, et al. (2001) Complete sequence characterization of the genome of the St Croix River virus, a new Orbivirus isolated from cells of Ixodes scapularis. J Gen Virol 82: 795–804.

6. Belaganahalli MN, Maan S, Maan NS, Nomikou K, Pritchard I, et al. (2012) Full Genome Sequencing and Genetic Characterization of Eubangene Virus Identify Pata Virus as a Distinct Species within the Genus Orbivirus. PLoS One 7(3): e31911. doi: 10.1371/journal.pone.0031911.

7. Roy P (2007) Fields Virology, 5th Edition. In Knipe DM, Howley PM, editors. Orbiviruses: Virus Structure. Wolters Kluwer-Lippincott Williams & Wilkins: Philadelphia. pp. 1976.

8. Mertens PPC, Diprose J, Maan S, Singh KP, Attou H, et al. (2004) Bluetongue virus replication, molecular, and structural biology. Vet Ital 40(4): 426–437.

9. Belhouachet M, Jaafar FM, Tesh R, Grimes J, Maan S, et al. (2010) Complete sequence of Great Island virus and comparison with the T2 and outer-capped proteins of Kemerovo, Lipovnik and Tribec viruses (genus Orbivirus, family Reoviridae). J Gen Virol 91: 2985–2993.

10. Belaganahalli MN, Maan S, Maan NS, Tesh R, Attou H, et al. (2011) Umatilla Virus Genome Sequencing and Phylogenetic Analysis: Identification of Stretch Lagoon Orbivirus as a New Member of the Orbivirus species. PLoS One 6(8): e23605. doi: 10.1371/journal.pone.0023605.

11. Li MH, Fu SH, Chen WX, Wang HY, Guo YH, et al. (2011) Genotype V Japanese Encephalitis Virus Is Emerging. PLoS Negl Trop Dis 5(7): e1231. doi: 10.1371/journal.pntd.0001231.

12. Li YX, Li MH, Fu SH, Chen WX, Liu QQ, et al. (2011) Japanese encephalitis, China. Emerg Infect Dis 17(5): 934–936. doi: 10.3201/eid1705.101417.

13. Wang J, Zhang H, Sun X, Fu S, Wang H, et al. (2011) Distribution of mosquitoes and mosquito-borne arboviruses in Yunnan Province near the China-Myanmar-Laos border. Am J Trop Med Hyg 85(5): 738–746. doi: 10.4269/ajtmh.2011.10-0294.
14. Wang D, Coscoy L, Zylberberg M, Avila PC, Boushey HA, et al. (2002) Microarray-based detection and genotyping of viral pathogens. Proc Natl Acad Sci U S A 99(24): 15687–15692.
15. Wang D, Urisman A, Liu YT, Springer M, Ksiazek TG, et al. (2003) Viral discovery and sequence recovery using DNA microarrays. PLoS Biol 1(2): E2.
16. Zhao G, Krishnamurthy S, Cai Z, Popov VL, Travassos da Rosa AP, et al. (2013) Identification of Novel Viruses Using VirusHunter—An Automated Data Analysis Pipeline. PLoS One 8(10): e78470. doi: 10.1371/journal.pone.0078470.
17. Attoui H, Billoir F, Biagini P, de Micco P, de Lamballerie X (2000) Complete sequence determination and genetic analysis of Banna virus and Kadipiro virus: proposal for assignment to a new genus (Scadornavirus) within the family Reoviridae. J Gen Virol 81(Pt 6): 1507–1515.
18. Matthijnssens J, Ciarlet M, McDonald SM, Attoui H, Bányai K, et al. (2011) Uniformity of rotavirus strain nomenclature proposed by the Rotavirus Classification Working Group (RCWG). Arch Virol 156(8): 1397–1413. doi: 10.1007/s00705-011-1006-z.
19. Grimes JM, Burroughs JN, Gouet P, Diprose JM, Malby R, et al. (1998) The atomic structure of the bluetongue virus core. Nature 395(6701): 470–478.
20. Gouet P, Diprose JM, Grimes JM, Malby R, Burroughs JN, et al. (1999) The highly ordered double-stranded RNA genome of bluetongue virus revealed by crystallography. Cell 97(4): 481–490.
21. Cowled C, Merville L, Weir R, Walsh S, Hyatt A, et al. (2007) Genetic and epidemiological characterization of Middle Point orbivirus, a novel virus isolated from sentinel cattle in northern Australia. J Gen Virol 88(Pt 12): 3413–3422.