Genetic diversity of *Usutu virus*

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SUMMARY

Usutu virus is a mosquito-borne virus first isolated from *Culex naevei* in South Africa in 1959. The first emergence of *Usutu virus* outside Africa was recorded in Austria. Here, a phylogenetic analysis targeting the E5 and NS5 genes was carried out on the viral strains circulating in Europe. The NS5 gene tree showed two main clades, one of which included the Italian sequences. In the E gene tree all sequences grouped into the same main clade, with sequences from Austria divided into two separate clusters. Only sites under negative selective pressure were found in E and NS5 proteins. The results suggest that *Usutu virus* circulating in Europe has a degree of genetic diversity higher than expected and that infection may arise from different sources.

KEY WORDS: Usutu, Phylogeny, Evolution

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INTRODUCTION

Usutu virus (USUV) is a member of the mosquito-borne cluster within the *Flavivirus* genus. USUV is closely related to important human pathogens such as *Japanese encephalitis virus* (JEV), *Murray Valley encephalitis virus* (MVEV), *Dengue virus* (DENV), *Yellow fever virus* (YFV), *Saint Louis encephalitis virus* (SLEV), and *West Nile virus* (WNV) (Bakonyi *et al.,* 2004; Bakonyi 2005; Buckley *et al.,* 2006). The virus was first isolated from *Culex naevei* in South Africa in 1959 (Williams MC *et al.,* 1964). The first emergence of USUV outside Africa was recorded in Austria (2001-2005) where it caused fatalities in wild blackbirds (*Turdus merula*) and captive great grey owls (*Strix nebulosa*) (Chvala *et al.,* 2007; Weissenböck *et al.,* 2002; Weissenböck *et al.,* 2003). Then, the virus spread to Hungary (Bakonyi *et al.,* 2007) and Italy (Mannarolla *et al.,* 2010) causing outbreaks. Although closely related to human pathogens, such as JEV and WNV, human disease caused by USUV has not been unequivocally reported, although USUV was isolated from a patient with fever and rash in Africa (Adam *et al.,* 2007), whereas viral RNA was detected in a patient with rash in Austria (Weissenböck *et al.,* 2004). A neuroinvasive USUV infection in a patient with diffuse large B cell lymphoma (Pecorari *et al.,* 2009), and in a patient who received an orthotropic liver transplant (Cavrini *et al.,* 2009) has also been described in Italy.

Diagnosis requires confirmation by demonstration of USUV, either by conventional or molecular methods or by the detection of viral signals in tissue sections (Chvala *et al.,* 2004). A specific vaccination is currently unavailable and the idea that another flavivirus vaccine, such as the WNV vaccine, induces cross protection to USUV seems rather unlikely (Johnson *et al.,* 2005; Okeson *et al.,* 2007). From the vaccine strategy point of view, it is important to mention that the Spanish USUV differs genetically from the one currently

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dispersing in Central Europe being more closely related to the African USUV isolates based on sequence similarity (Busquets *et al.,* 2008). However, in-depth studies on the genetic variability and phylogeny of these viruses have not been carried out so far. Current USUV outbreaks seem to be strongly limited to certain areas in Austria, Hungary, Italy, and Switzerland (Bakonyi *et al.,* 2007, Dorrestein *et al.,* 2007, Mannarolla *et al.*, 2010). Nevertheless, detection of the virus in mosquitoes and serological reactivity in wild birds has also been demonstrated in the Czech Republic, Great Britain, Germany, Poland and Spain (Vazquez *et al.,* 2010). In view of the spread of USUV through Central Europe, phylogenetic and evolutionary analyses seem to be essential to better understand the epidemiology and the distribution of the virus for vaccine and prevention strategy. The aim of this study was to gather sequence information available for USUV from the GenBank database in order to investigate the genetic diversity of this emerging pathogen and to get deeper insight into the phylogenetic relationship existing among viruses circulating in Europe.

MATERIAL AND METHODS

In this study two different datasets were analyzed: the first one included 23 sequences from the polyprotein E gene fragment (nt 1180 to 1583), while the second one included 21 sequences from the NS5 gene (nt 9214 to 9309). Positions were numbered according to the reference sequence AY453412. All the sequences were downloaded from the NCBI database (http://www.ncbi.nlm.nih.gov/Database/) and the relative accession numbers are reported in Table 1 A and B.

The phylogenetic analysis was carried out on a 404 bp fragment and 96 bp fragment for the first and the second dataset, respectively. All the sequences in each dataset were aligned using CLUSTAL X software (Thompson *et al.,* 1994); then manually edited with the Bioedit software (version 7.0.9) (Hall *et al.,* 1999). The phylogenetic tree was estimated using the PAUP* package (Swofford *et al.,* 2003). The Tamura-Nei (TrN93) and F81 models of nucleotide substitution were selected for E and NS5 gene fragments, respectively. For both datasets, Maximum Likelihood (ML) estimates of base composition and the shape parameter (α) of a gamma distribution (Γ) model of among-site rate variation using Modeltest v. 3.7 implemented in PAUP* was employed (Posada and Buckley, 1998; Posada and Buckley, 2004). Maximum Likelihood tree was

TABLE 1A - *USUV Env gene fragment sequences used in the study.*

Viral strains	*Country*	*Accession Numbers*
USU499-03	Austria	EF078296
USU338-04	Austria	EF078298
USU281-03	Austria	EF078294
USU502-03	Austria	EF078297
Vienna 2001	Austria	AY453411
1477	Germany	JF330418
BH65/11-02-03	Germany	HE599647
Budapest	Hungary	EF206350
USU450-03	Austria	EF078295
USU618-04	Austria	EF078299
USU623-04	Austria	EF078300
USU629-05	Austria	EF393681
USU-PR-m1/2010	Italy - Emilia-Romagna	JF834663
USU-RE-m6/2010	Italy - Emilia-Romagna	JF834671
USU-FC-b2/2010	Italy - Emilia-Romagna	JF834618
RA-09-Tm	Italy - Emilia-Romagna	JF331436
USU-MO-b1/2010	Italy - Emilia-Romagna	JF834683
UFU-FE-m2/2010	Italy - Emilia-Romagna	JF834682
USU-BO-m18/2010	Italy - Emilia-Romagna	JF834615
USU589-05	Austria	EF078301
USU588-05	Austria	EF393679
USU604-05	Austria	EF393680
SAAR-1776	South Africa	AY453412

estimated under the two different models using tree bisection-reconnection (TBR) branch swapping. The statistical robustness and reliability of the branching order within each phylogenetic tree were confirmed by bootstrap analysis using 1,000 replicates for the Neighbor-Joining (NJ) tree and by the Zero Branch Length Test for the ML tree (Swofford *et al.,* 2003).
For each data set the d_N/d_S rate (ω) was estimated by ML approach implemented in the HyPhy program (Pond and Muse, 2005). In particular, the global (assuming a single selective pressure for all branches) and local (allowing the selective pressure to change along every branch) models were compared by likelihood ratio test (LRT). Site specific positive and negative selections were estimated by two different algorithms: the fixed-effects likelihood approach (FEL) which fits an rate to every site and use likelihood ratio to test if $d_N \neq d_S$; and a random effect likelihood (REL), a variant of the Nielsen-Yang approach (Yang and Nielsen, 1998), which assumes there exists a discrete distribution of rates across sites and allows both d_S and d_N to vary site-by-site independently (Kosakovsky Pond and Frost, 2005). Sites under selective pressure were selected assuming a p value of ≤0.1 or a posterior probability of ≥0.9. Some analyses were carried out using the Web based interface Datamonkey (http://www.datamonkey.org/) (Kosakovsky Pond and Frost, 2005). For the evolutionary analysis, the AY453412 reference sequence was used to trace the exact position of the amino acids found under positive selection for the E and NS5 gene fragments, respectively. SNAP software (Korber, 2000) for the average of d_N/d_S rate was also used.

TABLE 1B - *USUV NS5 gene fragment sequences used in the study*

Viral strains	*Country*	*Accession Numbers*
Vienna 2001	Austria	AY453411
Budapest	Hungary	EF206350
BH65/11-02-03	Germany	HE599647
TV1-09-Tm	Italy - North Eastern	JF331432
VE-09-Tm	Italy - North Eastern	JF331433
UD-09-Tm	Italy - North Eastern	JF331435
RA-09-Tm	Italy - North Eastern	JF331436
MO-09-Hu	Italy - North Eastern	JF331434
TV3-09-Tm	Italy - North Eastern	JF331437
TV2-09-Tm	Italy - North Eastern	JF331431
USU-BO-1/2009	Italy - Emilia-Romagna	HM138707
USU-FE-1/2009	Italy - Emilia-Romagna	HM138715
USU-BO-10/2009	Italy - Emilia-Romagna	HM138718
USU-MO-m4/2010	Italy - Emilia-Romagna	JF834596
USU-RE-m2/2010	Italy - Emilia-Romagna	JF834585
USU-FE-m7/2010	Italy - Emilia-Romagna	JF834564
USU-BO-m16/2010	Italy - Emilia-Romagna	JF834558
USU-BO-7/2009	Italy - Emilia-Romagna	HM138714
USU-RA-m3/2010	Italy - Emilia-Romagna	HM834581
SAAR-1776	South Africa	AY453412

RESULTS

The phylogenetic analysis for the E gene fragment showed that all the European sequences grouped in the same main clade. However, sequences from Austria divided into two separate clusters statistically supported by 75% and 92% bootstrap values, respectively. The minor cluster included three sequences identified in Vienna in 2005 carrying one amino acid substitution at position 419 (Met to Val). The outgroup was represented by the oldest sequence isolated from South Africa (Figure 1). The phylogenetic tree for the NS5 gene fragment showed two main clades. The first one included only the Italian sequences identified in mosquitos from the Emilia-Romagna region by Calzolari *et al.* (hereafter named "Emilia-Romagna" sequences) and it was statistically supported (94% bootstrap value), whereas the second one included eight Italian sequences reported by Savini *et al.* (hereafter named "North Eastern Italy" sequences) and identified in blackbirds (Veneto, Friuli and Emilia-Romagna regions), humans (Emilia-Romagna region) and

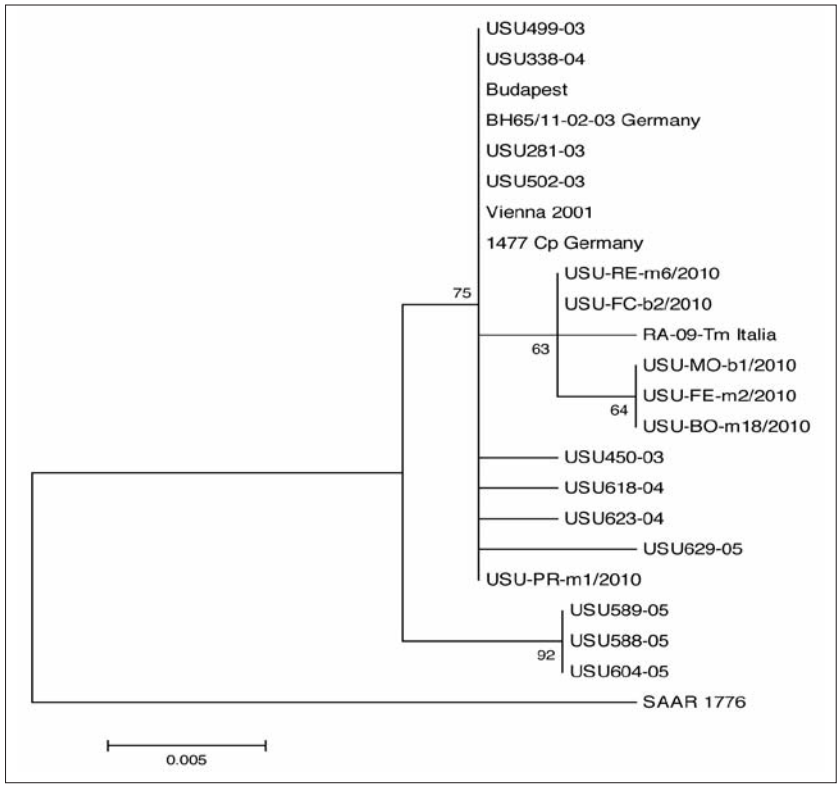

FIGURE 1 - *Maximun likelihood phylogenetic analysis of polyprotein E gene fragment sequences. The data set included 23 sequences from different countries downloaded from the NCBI database. The tree was rooted by using the oldest isolated sequence from South Africa. Branch lengths were estimated with the best fitting nucleotide substitution model according to a hierarchical likelihood ratio test, and were drawn to scale with the bar at the bottom indicating 0.005 nucleotide substitutions per site. Significant bootstrap values are indicated.*

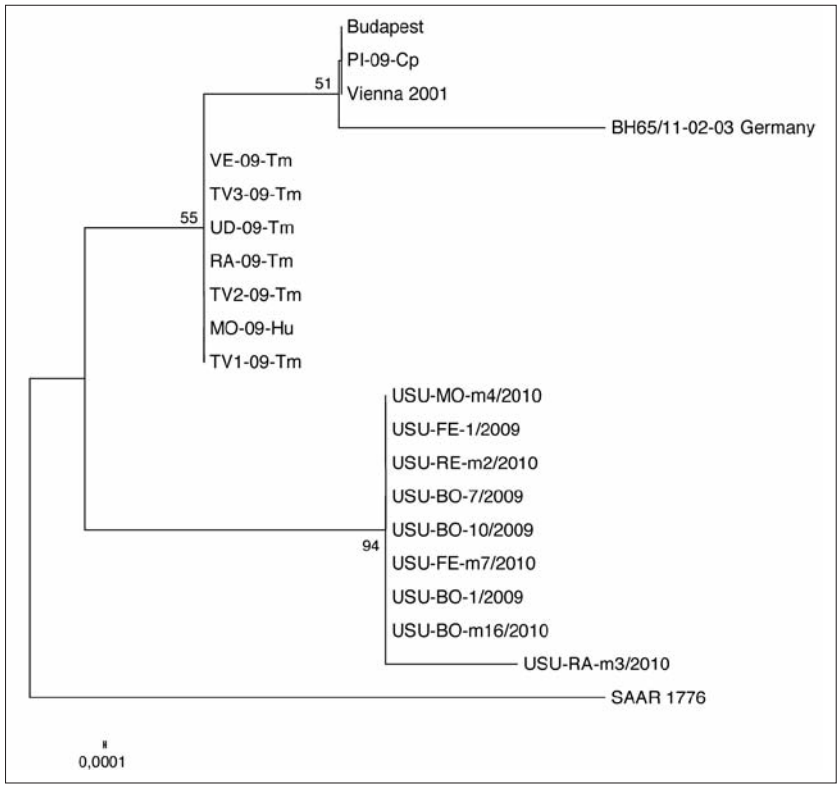

FIGURE 2 - *Maximun likelihood phylogenetic analysis of NS5 gene fragment sequences. The data set included 21 sequences from different countries downloaded from the NCBI database. The tree was rooted by using the oldest isolated sequence from South Africa. Branch lengths were estimated with the best fitting nucleotide substitution model according to a hierarchical likelihood ratio test, and were drawn to scale with the bar at the bottom indicating 0.0001 nucleotide substitutions per site. Significant bootstrap values are indicated.*

TABLE 2A - *Selection analysis for USUV polyprotein precursor gene (envelope protein) using HYPHY software.*

Negatively selected sites* (ω for sites <1) HYPHY software	403 (G); 414 (T); 417 (C); 445 (T); 486 (A); 490 (G); 518 (R); 577 (V); 595 (V); 613 (R); 643 (G); 691 (A); 707 (Y); 727 (S); 776 (T)

*Negatively selected sites are numbered according to amino acid position of USUV isolate SAAR-1776 complete genome (accession number AY453412). The amino acid present at each site under negative selection is given between brackets.

TABLE 2B - *Selection analysis for USUV NS5 gene using HYPHY software.*

Negatively selected sites* (ω for sites <1) HYPHY software	3020(K); 3021(R); 3022(E); 3029(G); 3051(A); 3077(K); 3088(H); 3091(G); 3095(A); 3098(T); 3103(T).

*Negatively selected sites are numbered according to amino acid position of USUV isolate SAAR-1776 complete genome (accession number AY453412). The amino acid present at each site under negative selection is given between brackets.

mosquitoes (Tuscany region) together with one Austrian, one Hungarian and one German sequence. This clade was not statistically supported. Even in this case, the outgroup was represented by the South African strain (Figure 2). Selection pressure analysis through computation of the ratio of non-synonymous (dN) to synonymous (dS) substitutions per site dN/dS (ω) revealed no positive selected sites but abundant negative sites under negative selective pressure, for both E and NS5 proteins. Indeed, eleven and fifteen negatively selected sites for E and NS5 protein were confirmed by both REL and FEL analysis (Table 2 A and B). This protein stability was supported by SNAP analysis that reported for the dN/dS ratio an average value of 0.032 and 0.037 for NS5 and E, respectively.

DISCUSSION

The USUV sequences published in GenBank and reporting homologous regions are scarce and, apart for a few full genome sequences of representative isolates, limited to the E and NS5 genes. Two datasets including available USUV E and NS5 gene sequences were analysed in this study. The analysis of the E gene dataset was performed on a region of 404 bp and homologous available sequences were from Austria (Chvala *et al.,* 2007), Hungary, Germany and Emilia-Romagna (Calzolari *et al.,* 2009). The E gene codes for the envelope protein, which is considered involved in neurovirulence and neuroinvasiviness of Flavivirus (Botha *et al.,* 2008; Beasley, 2009). The phylogenetic tree obtained using this marker (Figure 1) seems to confirm the scenario depicted by other Authors (Chvala *et al.,* 2007; Mannarolla *et al.,* 2010; Steinmetz *et al.,* 2011) who suggested a single introduction of USUV from Africa to Europe (Austria, 2001). Then the virus was able to establish an efficient mosquito-wild birds cycle by an overwintering mechanism and re-emerged in the following years both in Austria and in surrounding countries (Hungary, Germany, Italy). However, our analysis showed that three E gene sequences, identified in Austria in 2005, form a separate cluster, statistically supported by 92% bootstrap value, from the other European sequences. Looking at the sequence alignment, these Austrian sequences appear to carry one amino acid change at position 419 from Met to Val and two non coding nucleotide mutations compared to the Vienna 2001 strain. This finding could be explained by E gene mutations occurring in the original Vienna 2001 strain, as higher sequence diversity would be expected if a re-introduction of a different strain from Africa would have occurred (Chvala *et al.,* 2007).

To obtain a complete picture of USUV diversity based on NS5 gene, we used a data set aiming at maximizing the number of NS5 sequences for phylogenetic analysis. Sequence alignment showed an overlapping region of 96 nt which allowed to include in the analysis all representative NS5 sequences available in GenBank. The resulting tree

(Figure 2) showed a separate clade including the Emilia-Romagna sequences, but, unexpectedly, it was not the same clade previously reported by Savini *et al*. 2011 for North Eastern Italy sequences, which formed an additional separate cluster, closer to Central European strains. As reported by Savini *et al.* 2011, the strain identified in *Culex pipiens* from Tuscany in 2009 (PI-09-Cp) was the most similar among the Italian sequences to the Central European ones, clustering with the Austrian, Hungarian and German USUV strains. From our results, based on maximization of NS5 gene sequence analysis, we can infer that not two, but three different USUV strains have likely circulated in Italy in 2008-2010. Although, a short NS5 fragment was used and caution is mandatory, we consider the obtained phylogenetic tree reliable as the overall phylogenesis is consistent with previously reported NS5 tree topologies (Weissenböck et al., 2002; Savini et al., 2011) and concordant with the first NS5 tree based on a longer fragment. However, resolution of USUV phylogenesis is still suboptimal and strain classification is far from being unequivocally informative, probably due to the lack of published sequence and gaps in knowledge of epidemiology and biology of this emerging virus. Indeed, the phylogenetic tree based on a fragment of the E gene, was not able to discriminate the Emilia-Romagna strains from the Central European isolates. On the contrary, the NS5 tree clearly demonstrated a separate clade statistically supported by 94% bootstrap value. The group including sequences from North-Eastern Italy showed instead a lower bootstrap value (55%) which, together with their geographical position, does not completely exclude that these isolates do not represent a different USUV introduction, as previously hypothesised, but could have spread from Austria to North Eastern Italy gaining some degree of genetic diversity. This scenario may be supported by the fact that wild-bird migratory routes flying across Austria and North-Eastern Italy exist.

Selection pressure analysis showed negative selected sites in both E and NS5 proteins. These findings could reflect the ability of USUV to adapt to the new hosts and vectors balancing its pathogenic potential with herd immunity. This was showed quite clearly in Austria, where the impact of USUV on wild bird populations decreased after initial significant mortality due to the contact of the virus with an immunologically naïve population, similarly to what happened with West Nile Virus in North America. Our analysis demonstrates the tendency of USUV circulating in Europe to keep stable certain amino acid sites of E protein, one of the main pathogenic determinants of flaviviruses, and to establish effective mosquito-wild bird cycles, even though also other species might play a role in maintaining USUV in nature. The analysis showed that several amino acid sites are also under negative selection in the RNA dependent RNA polymerase, encoded by the NS5 gene. Nevertheless, this result may be less surprising because this region carries positions essential for its functional epitopes and, actually, NS5 is reported to exhibit the lowest level of sequence divergence among flaviviruses (Bakonyi et al., 2004).

In conclusion, the results suggest that USUV circulating in Europe has a greater degree of genetic diversity than expected and that probably different sources of infection may be hypothesized. Additional studies should be undertaken on wider genomic regions of USUV in order to identify new phylogenetic markers that would permit deeper insight into the molecular epidemiology of this emerging flavivirus.

REFERENCES

ADAM F., DIGUETTE J.-P. (2007). Virus d'Afrique [base de données]. Dakar: Institut Pasteur de Dakar. Centre collaborateur OMS de référence et de recherche pour les arbovirus et les virus de fièvres hémorrhagiques (CRORA). Available from: http://www.pasteur.fr/recherche/banques/CRORA

BAKONYI T., GOULD E.A., KOLODZIEJEK J., WEISSENBÖCK H., NOWOTNY N. (2004). Complete genome analysis and molecular characterization of Usutu virus that emerged in Austria in 2001: comparison with the South African strain SAAR-1776 and other flaviviruses. *Virol.* **328**, 301-310.

BAKONYI T., LUSSY H., WEISSENBÖCK H., HORNYÁK A., NOWOTNY N. (2005). In vitro host-cell susceptibility to Usutu virus. *Emerg. Infect. Dis.* **11**, 298-301.

BAKONYI T., ERDÉLYI K., URSU K., FERENCZI E., CSÖRGO T., LUSSY H., CHVALA S., BUKOVSKY C., MEISTER T., WEISSENBÖCK H., NOWOTNY N. (2007). Emergence of Usutu virus in Hungary. *J. Clin. Microbiol*. **45** (12), 3870-3874.

BEASLEY D.W. (2005). Recent advances in the molecular biology of west nile virus. *Curr. Mol. Med.* **5** (8), 835-50.

Botha E.M., Markotter W., Wolfaardt M., Paweska J.T., Swanepoel R., Palacios G., Nel L.H., Venter M. (2008). Genetic determinants of virulence in pathogenic lineage 2 West Nile virus strains. *Emerg. Infect. Dis.* **14** (2), 222-230.

Buckley A., Dawson A., Gould E.A. (2006). Detection of seroconversion to West Nile virus, Usutu virus and Sindbis virus in UK sentinel chickens. *Virol. J.* **3**, 71.

Busquets N., Alba A., Allepuz A., Aranda C., Ignacio Nuñez J. (2008). Usutu virus sequences in Culex pipiens (Diptera: Culicidae), Spain. *Emerg. Infect. Dis*. **14** (5), 861-863.

Calzolari M., Bonilauri P., Bellini R., Albieri A., Defilippo F., Maioli G., Galletti G., Gelati A., Barbieri I., Tamba M., Lelli D., Carra E., Cordioli P., Angelini P., Dottori M. (2010). Evidence of simultaneous circulation of West Nile and Usutu viruses in mosquitoes sampled in Emilia-Romagna region (Italy) in 2009. *PLoS One.* **5** (12), e14324

Cavrini F., Gaibani P., Longo G., Pierro A.M., Rossini G., Bonilauri P., Gerundi G.E., Di Benedetto F., Pasetto A., Girardis M., Dottori M., Landini M.P., Sambri V. (2009). Usutu virus infection in a patient who underwent orthotropic liver transplantation, Italy, August-September 2009. *Euro Surveill.* **17**, 14 (50), pii: 19448.

Chvala S., Kolodziejek J., Nowotny N., Weissenböck H. (2004). Pathology and viral distribution in fatal Usutu virus infections of birds from the 2001 and 2002 outbreaks in Austria. *J. Comp. Pathol.* **131** (2-3), 176-185.

Chvala S., Bakonyi T., Bukovsky C., Meister T., Brugger K., Rubel F., Nowotny N., Weissenböck H. (2007). Monitoring of Usutu virus activity and spread by using dead bird surveillance in Austria, 2003-2005. *Vet. Microbiol*. **122** (3-4), 237-245.

Dorrestein G.M., Crosta L., Steinmetz H.W., Bakony T., Nowotny N., Weissenböck H. (2007). Usutu virus activity is spreading in Europe. In:Proc. 9th European AAV Conference and 7th ECAMS Scientific Meeting. 7-8.

Hall T.A. (1999). Bioedit: a user friendly biological sequence alignment editor and analysis program for Windows 95/98 NT. *Nucleic. Acids. Symp. Ser*. **41**, 95-98.

Johnson C., Monath T.P., Kanesa-Thasan N., Mathis D., Miller C., Shapiro S., Nichols R., McCarthy K., Deary A., Bedford P. (2005). Exercise-induced serum enzyme elevations confounding the evaluation of investigational drug toxicity. Report of two cases in a vaccine trial. *Hum. Vaccin.* **1** (1), 24-29.

Korber B. (2000). HIV Signature and Sequence Variation Analysis. Computational Analysis of HIV Molecular Sequences,. Allen G. Rodrigo and Gerald H. Learn, eds. Dordrecht, Netherlands: Kluwer Academic Publishers. 55-72.

Kosakovsky Pond S.L., Frost S.D. (2005). Not so different after all: a comparison of methods for detecting amino acid sites under selection. *Mol. Biol. Evol.* **22** (5), 1208-1222.

Manarolla G., Bakonyi T., Gallazzi D., Crosta L., Weissenböck H., Dorrestein G.M., Nowotny N. (2010). Usutu virus in wild birds in northern Italy. *Vet. Microbiol*. **141** (1-2), 159-163.

Okeson D.M., Llizo S.Y., Miller C.L., Glaser A.L. (2007). Antibody response of five bird species after vaccination with a killed West Nile virus vaccine. *J. Zoo. Wildl. Med.* **38** (2), 240-244.

Pecorari M., Longo G., Gennari W., Grottola A., Sabbatini A., Tagliazucchi S., Savini G., Monaco F., Simone M., Lelli R., Rumpianesi F. (2009). First human case of Usutu virus neuroinvasive infection, Italy, August-September 2009. *Euro Surveill.* **17**, 14 (50). pii: 19446.

Pond S.K., Muse S.V. (2005). Site-to-site variation of synonymous substitution rates. *Mol. Biol. Evol,* **22** (12), 2375-2385.

Posada D., Crandall K.A. (1998). MODELTEST: testing the model of DNA substitution. *Bioinformatics*. **14** (9), 817-818.

Posada D., Buckley T.R. (2004). Model selection and model averaging in phylogenetics: advantages of akaike information criterion and bayesian approaches over likelihood ratio tests. *Syst. Biol.* **53** (5), 793-808.

Savini G., Monaco F., Terregino C., Di Gennaro A., Bano L., Pinoni C., De Nardi R., Bonilauri P., Pecorari M., Di Gialleonardo L., Bonfanti L., Polci A., Calistri P., Lelli R. (2011). Usutu virus in Italy: an emergence or a silent infection? *Vet. Microbiol.* **151** (3-4), 264-274.

Steinmetz H.W., Bakonyi T., Weissenböck H., Hatt J.M., Eulenberger U., Robert N., Hoop R., Nowotny N. (2011). Emergence and establishment of Usutu virus infection in wild and captive avian species in and around Zurich, Switzerland-genomic and pathologic comparison to other central European outbreaks. *Vet. Microbiol.* **148** (2-4), 207-212.

Swofford D., Sullivan J. (2003). Phylogeny inference based on parsimony and other methods with PAUP*. In: The phylogenetic handbook - A practical approach to DNA and protein phylogeny. (Salemi M, Vandamme AM, ed.) Cambridge University Press, New York. 160-206.

Thompson J.D., Higgins D.G., Gibson T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic. Acids Res.* 22(22), 4673-4680.

Vazquez A., Jimenez-Clavero M., Franco L., Donoso-Mantke O., Sambri V., Niedrig M., Zeller H., Tenorio A. (2011). Usutu virus: potential risk of human disease in Europe. *Euro Surveill*. **16** (31), pii: 19935.

YANG Z., NIELSEN R. (1998). Synonymous and nonsynonymous rate variation in nuclear genes of mammals. *J. Mol. Evol.* **46** (4), 409-418.

WILLIAMS M.C., SIMPSON D.I., HADDOW A.J., KNIGHT E.M. (1964). The isolation of west nile virus from man and of usutu virus from the bird-biting mosquito mansonia aurites (Theobald) in the entebbe area of Uganda. *Ann. Trop. Med. Parasitol.* **58**, 367-374.

WEISSENBÖCK H., KOLODZIEJEK J., URL A., LUSSY H., REBEL-BAUDER B., NOWOTNY N. (2002). Emergence of Usutu virus, an African mosquito-borne flavivirus of the Japanese encephalitis virus group, central Europe. *Emerg. Infect. Dis.* **8** (7), 652-656.

WEISSENBÖCK H., KOLODZIEJEK J., FRAGNER K., KUHN R., PFEFFER M., NOWOTNY N. (2003). Usutu virus activity in Austria, 2001-2002. *Microbes. Infect.* **5** (12), 1132-1136.

WEISSENBÖCK H., BAKONYI T., CHVALA S., NOWOTNY N. (2004). Experimental Usutu virus infection of suckling mice causes neuronal and glial cell apoptosis and demyelination. *Acta Neuropathol.* **108** (5), 453-460.