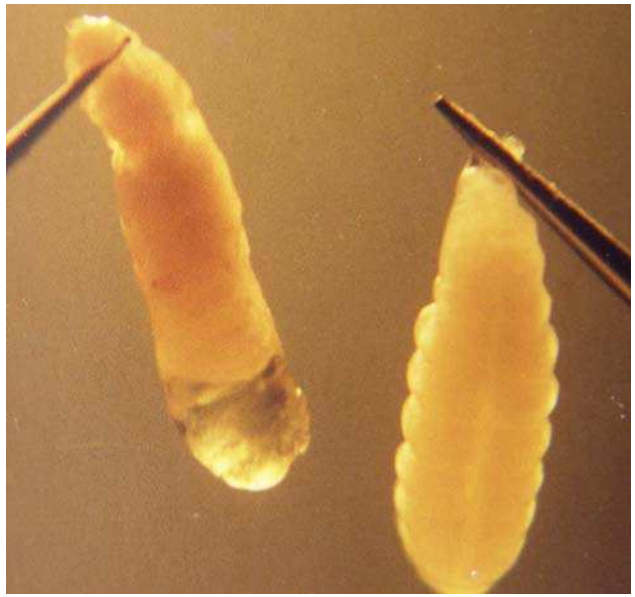
APPLICATION OF MULTIPLEX RT-PCR/RFLP FOR EARLY DETECTION AND GENETIC RELATIONSHIP RESEARCH OF SACBROOD VIRUS ON HONEYBEES IN VIETNAM

Presented by
Lê Quang Trung

BEE RESEARCH AND DEVELOPMENT CENTER
19 – Truckhe, Dong Da, HANOI, VIETNAM
Tel.: +84-4-38343185
Fax: +84-4-37733982

INTRODUCTION: SACBROOD VIRUS

- Three strains of Sacbrood virus (SBV):
  - SBV on *A. mellifera* (AmSBV)
  - Thai SBV on *A. cerana* (AcTSBV),
  - Chinese SBV on *A. cerana* (AcCSBV)
- SBV infects larvae of both the honeybee species resulting in failure to pupate and death



INTRODUCTION: SBV DISEASE

- Without treatment, SBV exists from year to year in infected beehives

- In Vietnam, SBV disease was found on *A. cerana* in 1974-1975 and on *A. mellifera* in 2006:

  - Loss of 7000 out of 74000 beehives of *A. cerana* during 1974-1975
  - Reduction in 40-80% of honey production of *A. mellifera* in 2008

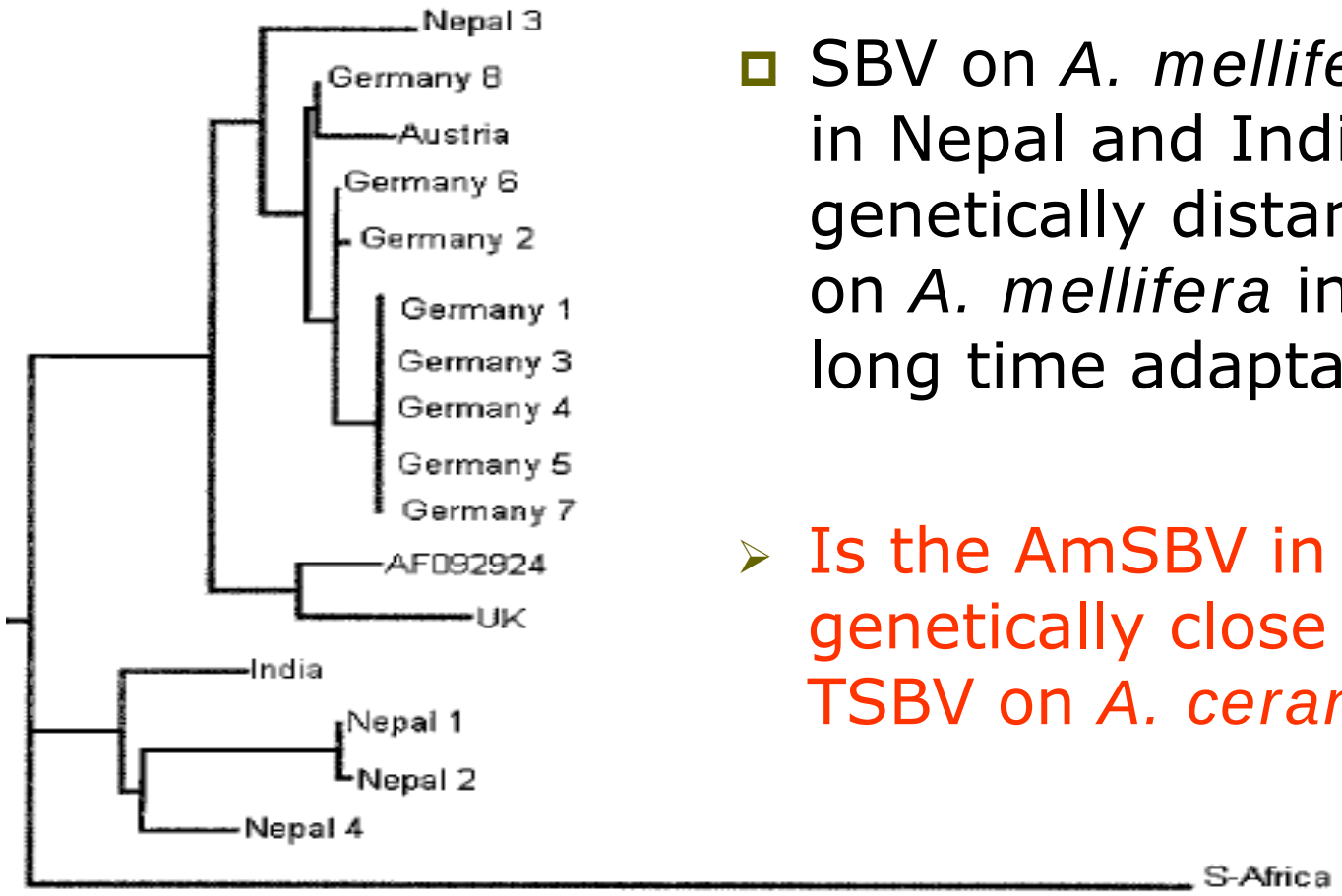
INTRODUCTION: EARLY DIAGNOSIS

- No antibiotics, medicaments or strategies for successful treatment of beehives with SBV disease.
  - Early diagnosis of SBV infection in beehives: more effectiveness of biological application or beekeeping management for SBV treatment.
- Difficulty in diagnosis of SBV at early stage of infection: workers remove infected larvae.

INTRODUCTION: DIAGNOSIS METHODS

- Traditional methods: electron microscopic identification, antigen detection such as enzyme-linked immuno-sorbent assay (ELISA)
  - low sensitivity and specificity or exhibition of nonspecific reactions
  - differentiation between virus types is difficult or impossible
- Molecular methods based on markers from RNA (cDNA) sequencing, RT-PCR, multiplex RT-PCR/RFLP for SBV detection
  - Genomes of AmSBV, AcCSBV and some loci of AcTSBV were completely sequenced and published on the Gene-bank: easier to design primers
  - Various successes are based on fragment length (FLPs) and Sequence polymorphism markers for SBV research.

INTRODUCTION: GENETIC RELATIONSHIP



Source: Grabensteiner et al. 2001
Clinic. Diag. Lab. Imm V8 (1):93–104

- SBV on *A. mellifera* (AmSBV) in Nepal and India is genetically distant from SBV on *A. mellifera* in Europe for long time adaptation

- Is the AmSBV in Nepal genetically close to CSBV or TSBV on *A. cerana* (AcCSBV)?

OBJECTIVES

- Application of multiplex RT-PCR/RFLP approach and analysis of published nucleotide sequences to find fragment length polymorphisms (FLPs) makers for early detection of SBV.
- Analysis of sequence polymorphisms from RT-PCR products (cDNA) and published nucleotide sequences to research genetic relationships between SBV strains.

MATERIALS

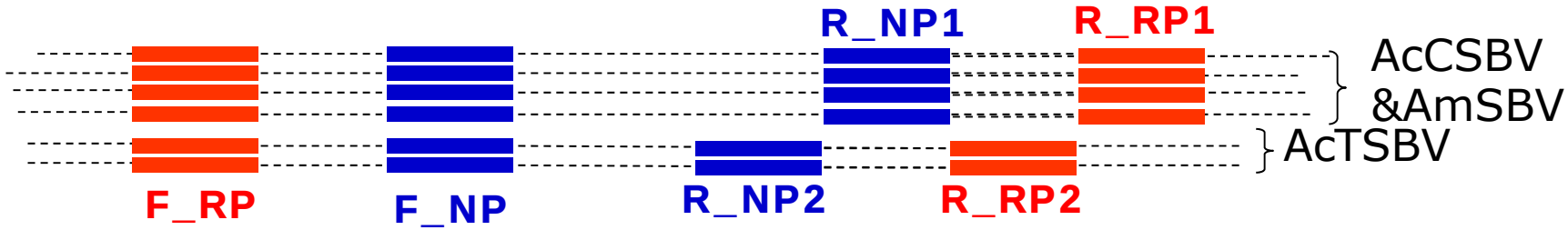
- Samples: Almost capped worker larvae in 5 *A. cerana* hives with no symptoms of sacbrood disease *

- 19 published sequences

Regions	Host	Codes	References
HoaBinh, VN	*A. c*	HoaBinhVN1-5	This research *
HaTay, VN	*A. c*	HaTayVN	Le *et al.*, 2003 *
HaiHung, VN	*A. c*	HaiHungVN	
India	*A. c*	AcTSBV	Rana và Rana, 2007
China	*A. c*	AcCSBV *(G)*	Zhang *et al.*, 2001
Anh	*A. m*	AF092924 *(G)*	Ghosh *et al.*,1999
India	*A. m*	India	Grabensteiner *et al.*, 2001
Nepal	*A. m*	Nepal1, 3	
England	*A. m*	UK	
Austria	*A. m*	Austria	
Germany	*A. m*	Germany1,6,7,8	

METHODS:RNA isolation,Primer design

- Isolation of RNA: as standardized by Grabensteiner *et al* (2001) using diethyl pyrocarbonate-treated water for SBV isolation and QIAmp viral RNA purification kit (Qiagen) for RNA extraction.
- Design of first round primers (RPs) and nested primers (NPs): multiple alignment published DNA sequences using Primer Designer Program in DNAMAN v.4.0



➡ A first round primer pair F_RP, R_NP1 for RT-PCR to detect AcCSBV and AmSBV and a pair F_RP, R_RP2 for AcTSBV detection.

➡ A nested primer pair F_RP, R_NP1 for PCR to check RT_PCR without products.

METHODS: MULTIPLEX RT-PCR, RFLPS

- Multiplex RT-PCR: reverse transcription and DNA amplification in one uninterrupted reaction using the Qiagen one-step RT-PCR kit (Qiagen) and 3 first round primers
- Nested PCR: RT-PCR product of sample(s) without RT-PCR products and 3 nested primers using kit of Promega.
- PCR-RFLPs:
  - Multiple alignment of published cDNA sequences of SBV to find digestion points of restricted enzyme (s)
  - Digestion of RT-PCR products (cDNA) with restricted enzyme (s) by incubation in right temperature and duration according to particular enzyme.

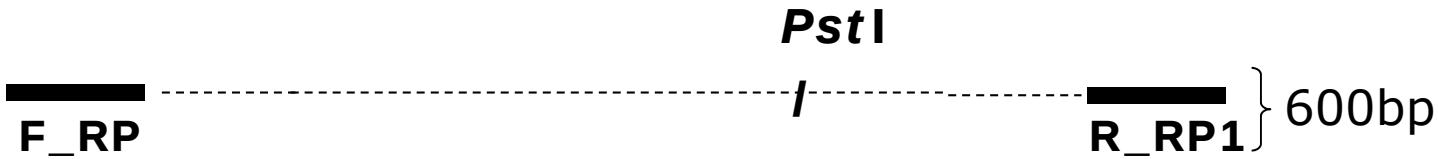
METHODS: SEQUENCE ANALYSIS

- Sequencing:
  - TA cloning: Ligation of RT-PCR products (cDNA) into pGEM-T vector using Promega's pGEM-T kit, and transformation in DH5α competent cells
  - Plasmid DNA extraction with The FavorPrep™ Plasmid Extraction Kit
  - Sequencing ligated DNA by Macrogen in Korea using M13 primers
- Sequence analysis: using program for neighbor joining with boostrap test of phylogeny in Mega v.3.1

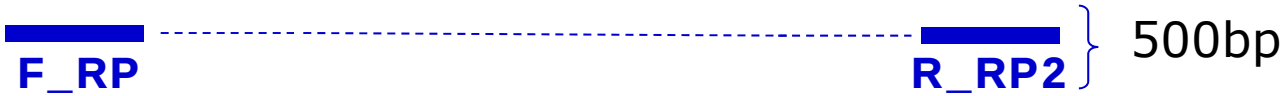
RESULTS: MOLECULAR MARKERS

- Fragment length polymorphisms markers for 3 SBV strains:
  - ✓ Evidence from Published sequences analysis
    - Markers for **AmSBV**: **600bp**

    - Markers for **AcCSBV**: **414bp and 186bp**



    - Markers for **AcTSBV: 500bp**

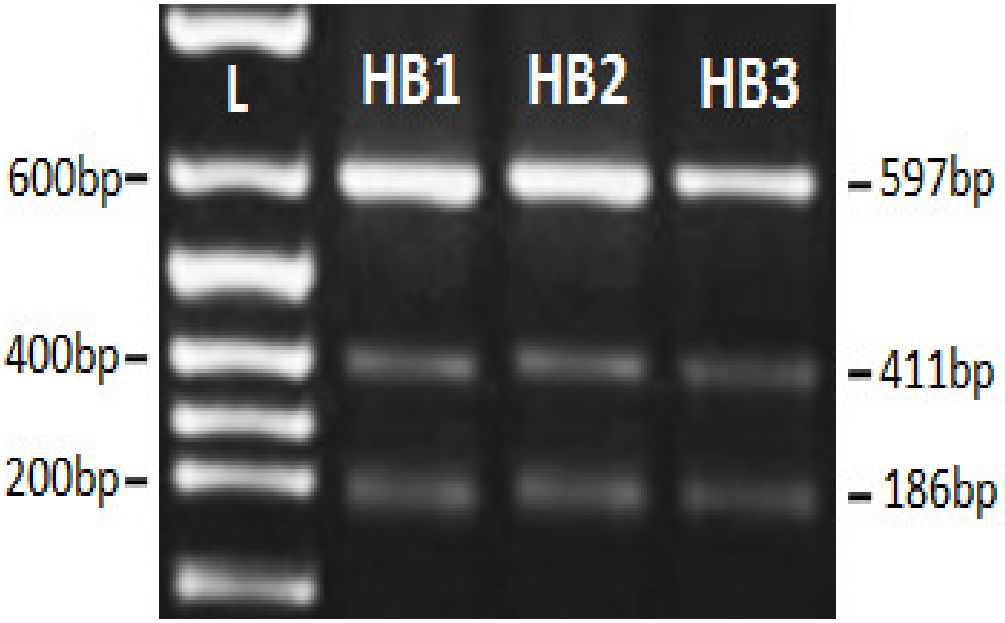


RESULTS: MOLECULAR MARKERS

- ✓ Evidence from multiplex RT-PCR/RFLP:

- RT-PCR with RPs and PCR with NPs: 3 of 5 sampled colonies of *A. cerana* infected with either AmSBV or AcCSBV: markers for both of about 600bp

- RFLPs by digestion with *Pst*I: 3 sampled colonies of *A. cerana* infected with AcCSBV: markers of 411bp and 186bp

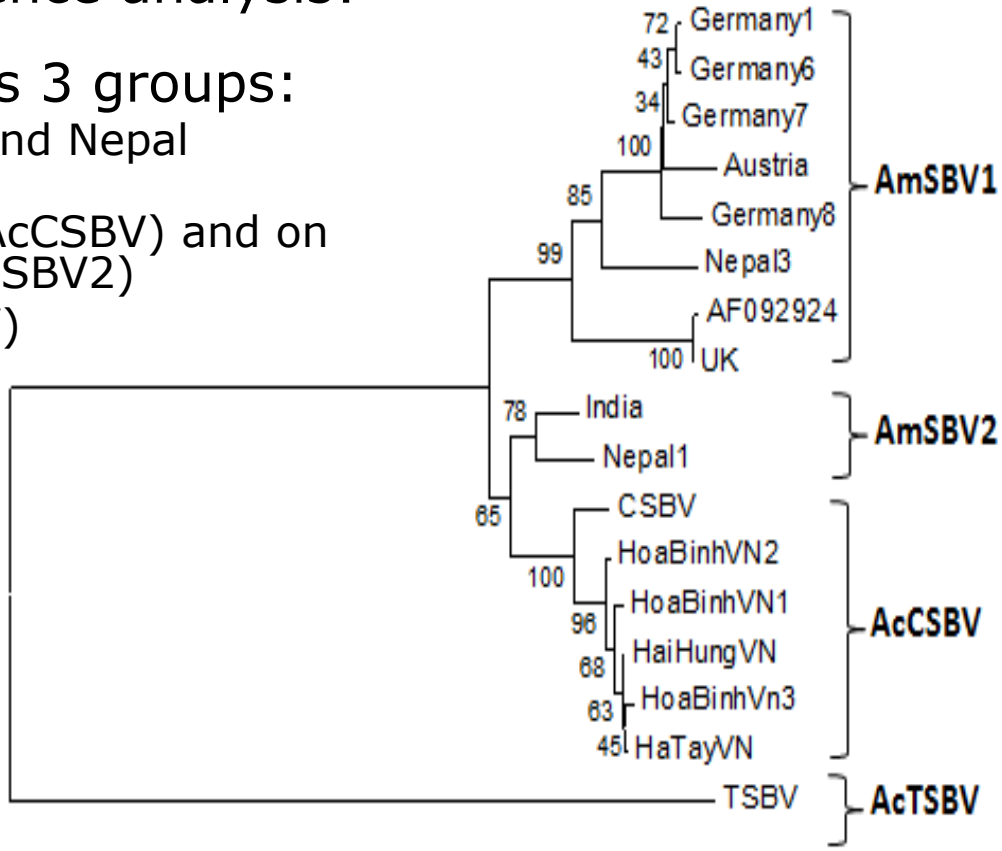


- Markers for **AcCSBV**: **411bp and 186 bp**

RESULTS: GENETIC RELATIONSHIPS

- SBV on *A. cerana* samples is CSBV (AcCSBV); SBV on *A. mellifera* in India and Nepal (AmSBV2) is closer to CSBV than SBV on *A. mellifera* (AmCSBV) in Europe.
  - ✓ Evidence from sequence analysis:

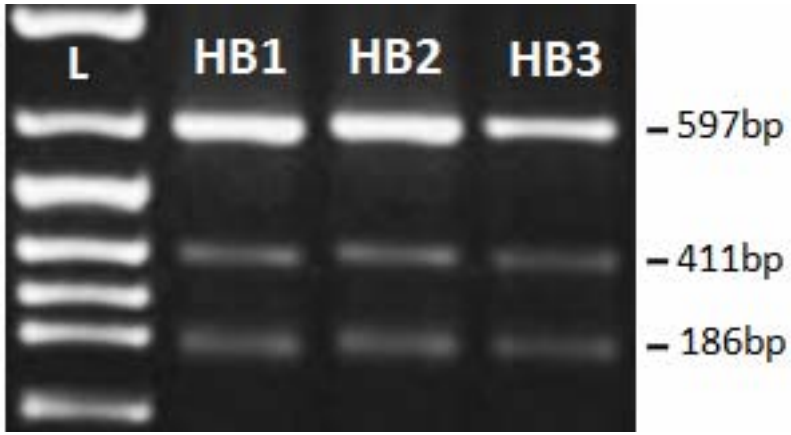
- Neighbour joining tree shows 3 groups:
  1) SBV on *A. mellifera* in Europe and Nepal (AmSBV1)
  2) SBV on *A. cerana* in Vietnam (AcCSBV) and on *A. mellifera* in Nepal, India (AmSBV2)
  3) Thai SBV on *A. cerana* (AcTSBV)
- SBV on *A. cerana* in Vietnam is close to CSBV on *A. cerana* in China
- AmSBV2 closer to AcCSBV than AmSBV
- AcTSBV genetically distant from other SBV strains

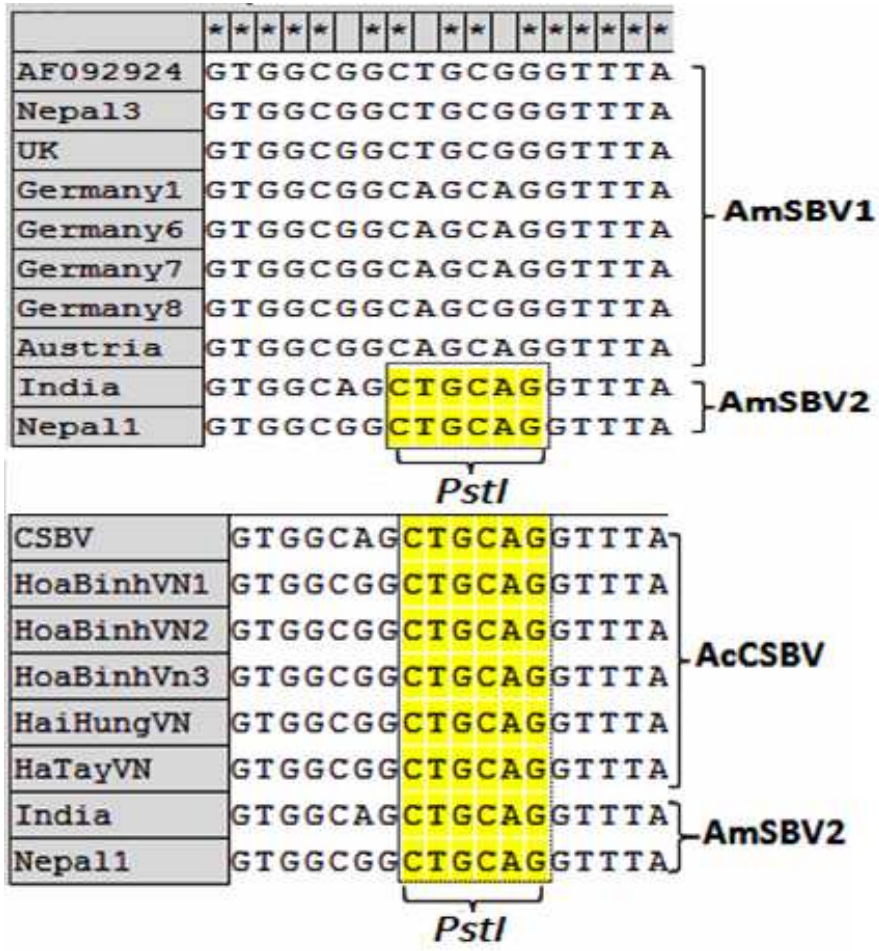


RESULTS: GENETIC RELATIONSHIPS

- Evidence from PCR-RFLP and sequence analysis:

- No *Pst*I point on sequence of markers for AmSBV1
- *Pst*I point on sequences of markers for both AcCSBV and AmSBV2



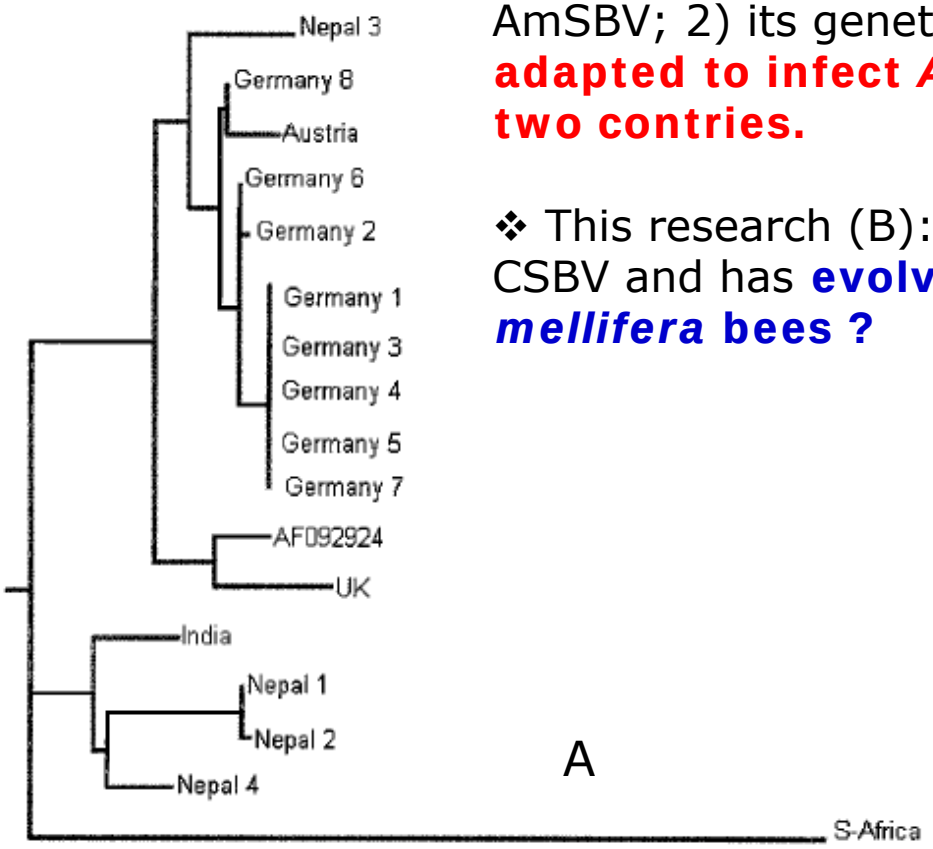


RESULTS: GENETIC RELATIONSHIPS

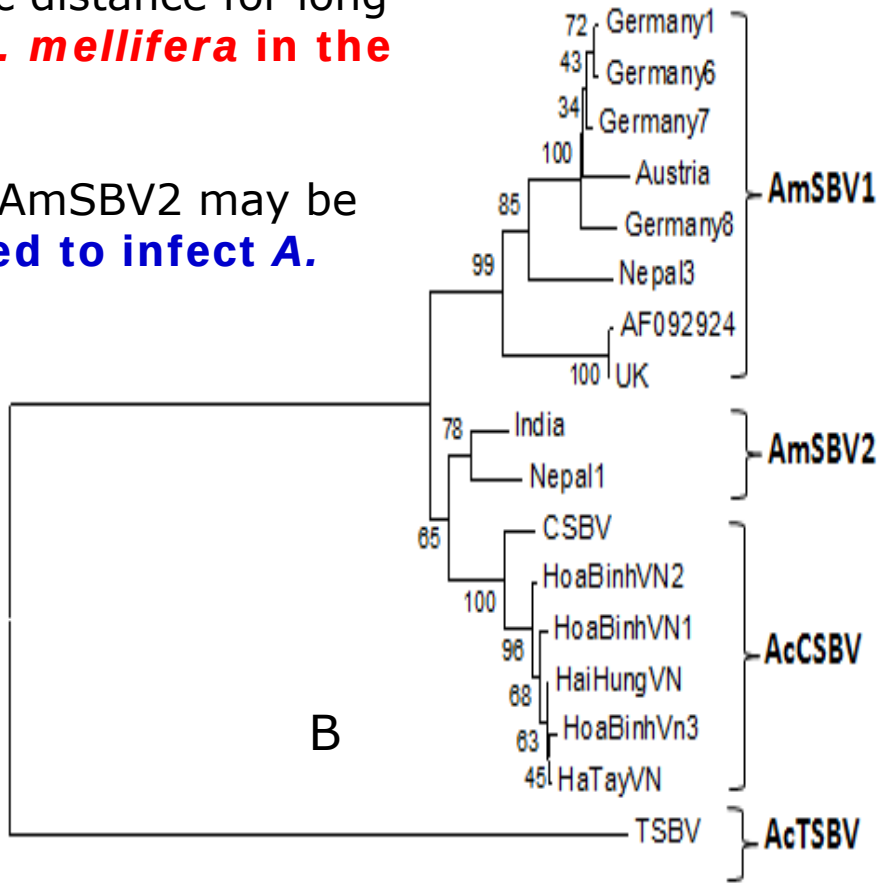
- Evidence from reference:

❖ Grabensteiner *et al.*, 2001 (A): 1) SBV on Am in Nepal and India (AmSBV2) is AmSBV; 2) its genetic distance for long-**adapted to infect *A. mellifera* in the two contries.**

❖ This research (B): AmSBV2 may be CSBV and has **evolved to infect *A. mellifera* bees ?**



A



B

Grabensteiner *et al.*, 2001

CONCLUSION AND RECOMENDATION

Conclusion:

- ☑ Multiplex RT-PCR/RFLP could be molecular tool to result in FLPs markers to detect 3 strains of SBVs on *A. cerana* and *A. mellifera*.
- ☑ Nucleotide sequence polymorphism from the multiplex RT-PCR products could be molecular markers to reveal genetic relationship among 3 SBV strains.

Recomendation

☞Multiplex RT-PCR/RFLP could be molecular tool to result in FLPs markers to detect 3 strains of SBVs on *A. cerana* and *A. mellifera*.


Thank you for you attention